Amphibian Sperm: Phylogeny and Fertilization Environment

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ABSTRACT

Sperm characteristics such as the structure and position of the acrosome, the design and ultrastructure of the flagellum, the presence/absence of a neck-piece and several other fine structural details are important diagnostic features which distinguish the three orders, families and species of lissamphibians. These sperm features are also important in phylogenetic inferences. It appears that the axoneme-undulating membrane-axial rod represents a plesiomorphic character. This pattern has been simplified to a single axoneme as a secondary reversal for external fertilization in many anurans or has developed as a double axoneme as in *Chiromantis xerampelina*. Sperm head and acrosome length are predictive in distinguishing between terrestrial and aquatic anuran fertilizers. Sperm motility is species specific among representatives of seven of the nine South African families and motile sperm which exhibit forward progression are the rule for aquatic fertilizing anurans. In contrast, the sperm of terrestrial fertilizing anurans are immotile in a wide range of physiological/culture media with osmotic concentrations varying from 10 to 300 mOsm/kg. The term ect-terrasperm is suggested as a new terminology for amphibians which exhibit the terrestrial mode of fertilization.

RÉSUMÉ

Spermatozoïdes des Amphibiens: phylogénie et environnement de la fécondation

Les caractéristiques du spermatozoïde telles que la structure et la position de l'acrosome, la forme et l'ultrastructure du flagelle, la présence ou absence d'un cou et plusieurs autres détails ultrastructuraux sont des critères de diagnostic qui distinguent les trois ordres, les familles et les espèces de Lissamphibiens. Les caractéristiques du spermatozoïde sont aussi importantes pour la compréhension de la phylogénie. Il semble que l'axonème avec membrane ondulante et fibre axiale représente un caractère plésiomorphe. Cette structure a été simplifiée en un axonème simple, comme réversion secondaire pour la fécondation externe, chez de nombreux Anoures, ou s'est développée en un axonème double comme chez *Chiromantis xerampelina*. La longueur de la tête du spermatozoïde ou de l'acrosome permet de distinguer de manière prédictive les Anoures à fécondation aquatique ou terrestre. La motilité du spermatozoïde est spécifique des espèces parmi les représentants de sept parmi les neuf familles d'Afrique du Sud, et les spermatozoïdes mobiles qui montrent une progression vers l'avant sont la règle pour les Anoures à fécondation aquatique. Par contre, les spermatozoïdes des Anoures à fécondation terrestre sont immotiles dans de nombreux milieux physiologiques et de culture avec des concentrations osmotiques variant de 10 à 300 mOsm/kg. Le terme ect-terraspermatozoïde est suggéré comme une nouvelle terminologie pour les Amphibiens qui possèdent le mode terrestre de fécondation.

VAN DER HORST, G., WILSON B., & CHANNING, A., 1995. — Amphibian sperm: phylogeny and fertilization environment. *In*: JAMIESON, B. G. M., AUSIO, J., & JUSTINE, J.-L. (eds), Advances in Spermatozoal Phylogeny and Taxonomy. *Mém. Mus. natn. Hist. nat.*, **16X** : 333-342. Paris ISBN : 2-85653-225-X.

Studies at both the light microscopic and the ultrastructural level suggest that sperm morphology relates to the mode of fertilization (internal versus external) [8, 10, 24] and is of importance in phylogenetic reconstruction [7, 10, 11]. Furthermore, sperm morphology assists in distinguishing between families [25] and even between genera [6] and closely related species [4, 9, 20, 23].

In their work on the *Hyla rubra* group, FOUQUETTE & DELAHOUSSAYE [7] regarded sperm structure as important in phylogenetic reconstruction, rather than an adaptation to the fertilization environment. LEE & JAMIESON [12] used phylogeny as an important basis for their work on the ultrastructure of myobatrachid frog sperm. In a later work, JAMIESON *et al.* [10] compared anuran and urodele sperm to that of *Ascaphus*. In this study they indicated which ultrastructural features of sperm could be considered primitive (plesiomorphic), associated with distinct advanced characteristics (autopomorphies), and derived features which were considered paedomorphic. It should be realized that sperm of an individual species may exhibit combinations of these features and that this may complicate interpretation of their phylogenetic position. In addition the latter authors [10] also indicated the relationship of these features to the fertilization biology of amphibians and suggested that the general trend towards simplification of sperm in anurans is a result of secondary reversion to external fertilization.

Among vertebrates, amphibians represent the widest range of "fertilization environments". Internal fertilization is evident in most salamanders and in all caecilians. In anurans such as *Ascaphus* internal fertilization takes place and their sperm are classified as introsperm by JAMIESON & ROUSE [11] and modified sperm by FRANZÉN [8]. External fertilization is the rule for most anurans and their sperm can be classified as ect-aquasperm [11] or in the terminology of FRANZÉN [8] as primitive. The terminology "primitive" used by FRANZÉN [8] is, however, confusing in terms of anurans since they are mainly external fertilizers but have modified sperm. Furthermore, the mode of external fertilization varies among anurans. Direct sperm deposition on the eggs occurs in *Arthroleptella lightfooti* and *Breviceps gibbosus* and sperm only swim through the mucous/egg jelly surrounding of the egg but are not substantially in contact with external fluids or fluids that are not largely from a biological origin of the species themselves. Species exhibiting this pattern will here be referred to as terrestrial fertilizers (TF). In *Bufo* spp. and *Xenopus laevis*, however, sperm are ejaculated in fresh water and sperm actually swim through an external medium to reach the eggs. Species exhibiting this pattern will be referred to as aquatic fertilizers (AF) [24]. It is therefore evident that amphibians represent an ideal model to investigate the relationship of sperm structure to their different fertilization environments.

It can furthermore be expected that sperm shape (particularly the head) and flagellar beat will largely determine the swimming behaviour of sperm. The importance of sperm motility is that it represents a summation of form, mitochondrial function, membrane integrity, exchange of important ions such as calcium and unmasking of receptors. It may be possible that specific sperm motility patterns are associated with mode of fertilization and also with species specific motility patterns.

Our aims in this investigation are to compare sperm structure in the urodeles, caecilians and anurans with special reference to seven of the nine South African anuran families. Here fertilization biology is contrasted with features that are important in establishing phylogenetic relationships. Furthermore, we investigate quantitatively sperm motility patterns in anurans as a function of their fertilization biology. In this part of the investigation we study quantitative sperm motility in representative examples of frogs exhibiting either the terrestrial mode of fertilization or the aquatic mode of fertilization. Lastly, we ascertain whether sperm motion is species specific in anurans.

MATERIALS AND METHODS

General. All comparisons that relate to sperm of the urodeles are extracted from the literature (Table 1). Sperm of only six caecilians have been described in the literature [16, 17, 18, 22], including only one paper on ultrastructure, that of *Typhlonectes natans* [22]. The data on anuran sperm in this paper refer to our original research and representatives of seven of the nine South African families are included. Table 1 refers to lissamphibian species used for comparisons in this investigation.

Capture of animals All anurans used in this study were captured at night during the peak of their breeding season. Animals were transported to the laboratory, were anaesthetized with MS222, dissected and sperm aspirated from the testes within 48 hours of capturing.

Sperm aspiration Testes were removed and all superficial blood vessels and connective tissue were removed. One testis of each specimen was put between two glass slides and squashed. Mainly intact sperm and testicular fluid could be aspirated by means of a micro-pipette. Five µl of this sperm suspension was used for scanning electron microscopy, 5 µl for motility studies and 5 µl for making sperm smears. The other testis was cut into smaller blocks and prepared for transmission electron microscopy.

SEM and TEM preparation Sperm suspension obtained as indicated above was processed for scanning electron microscopy according to the method of VAN DER HORST *et al.* [21]. Small testes blocks were fixed in Sørenson's phosphate buffered glutaraldehyde (2,5%) and post-fixed in 1% osmium tetroxide in the same buffer. Routine processing followed and the material was embedded in Spurr's resin.

Sperm dimensions Sperm were incubated in a nigrosin-eosin solution, sperm smears made and used for measurement of sperm dimensions (acrosome length, sperm head length, sperm head width) using a Kontron image analyzer.

Sperm motility Distilled water, pond water and various physiological media were used at different concentrations (0-300 mOsm/Kg) to establish the ideal medium for activating anuran sperm. HAM's F10 culture medium at 30 mOsm/kg provided the best medium and also preserved motility in most species for several hours [24]. Five ml of sperm suspension was placed in a microscopic bath containing 1 ml of HAM's F10 medium and sperm motion was recorded by means of a video-camera. The images were later replayed and detailed sperm motion characteristics analysed in the fully automated mode by means of computer aided sperm motility analysis (CASMA) utilizing the Sperm Motility Quantifier (SMQ, Wirsam Scientific, South Africa). The curvilinear velocity (VCL), the straight line velocity (VSL), the linearity (LIN), the average path velocity (VAP), amplitude of lateral head displacement (ALH) and dance (DNC = VCL x ALH) were measured. Fig. 1 diagrammatically depicts a typical sperm swimming trajectory and shows the applicable terminology.

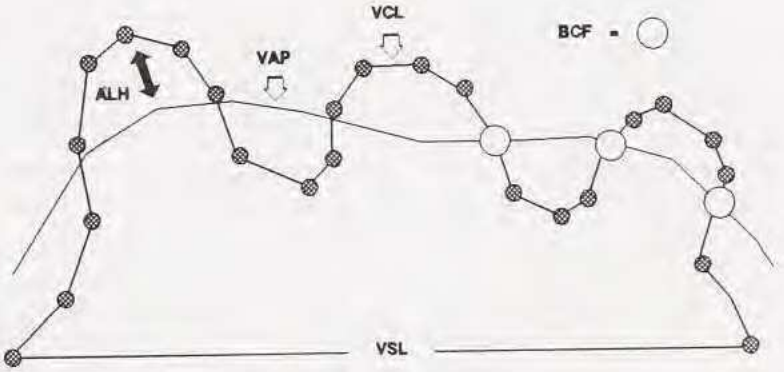


FIG. 1. — Sperm track indicating relevant terminology. VCL, curvilinear velocity; VSL, Straight line velocity; ALH, Amplitude of lateral head displacement; BCF, Beat cross frequency. LIN = Linearity = VSL/VCL.

TABLE 1. — List of species used for drawing comparisons among the Lissamphibia. Only South African anurans which represent our original research are listed. (TF) = Terrestrial fertilizers and (AF) = aquatic fertilizers. Other Anurans are used for comparison in text.

Order	Family	Species	Reference
Urodela	Plethodontidae	*Plethodon albagula*	[10]
	Amphiumidae	*Amphiuma tridactylum*	[2]
	Salamandridae	*Triturus viridescens*	[3]
		Pleurodeles waltlii	[14]
	Proteidae	*Necturus maculosus*	[1]
	Cryptobranchidae	*Cryptobranchus alleganiensis*	[1]
	Ambystomidae	*Ambystoma maculatum*	[3]
Gymnophiona	Ichthyopiidae	*Ichthyopis glutinosus*	[16, 17]
		Uraeotyphlus narayani	[18]
	Caeciliidae	*Siphonops annulatus*	[16]
		Gegeneophis carnosus	[18]
	Typhlonectidae	*Typhlonectes natans*	[22]
		Chthonerpton indistinctum	[5]
Anura	Bufonidae	*Bufo rangeri* (AF)	[24, Present study]
	Hemisotidae	*Hemisus marmoratus* (TF)	[24, Present study]
	Hyperoliidae	*Hyperolius horstocki* (AF)	[24, Present study]
		Semnodactylus wealii (AF)	[24, Present study]
		Leptopelis flavomaculatus (TF)	[24, Present study]
	Mycrohylidae	*Breviceps gibbosus* (TF)	[24, Present study]
	Pipidae	*Xenopus laevis* (AF)	[19, 24, Present study]
	Ranidae	*Arthroleptella lightfooti* (TF)	[24, Present study]
		Pyxicephalus adspersus (AF)	[24, Present study]
		Rana fuscigula (AF)	[24, Present study]
		Strongylopus grayii (AF)	[24, Present study]
		Tomopterna delalandii (AF)	[24, Present study]
	Rhacophoridae	*Chiromantis xerampelina* (TF)	[24, Present study]

RESULTS

Structural considerations of South African anurans

Figs. 2 to 8 are scanning and transmission electron micrographs which represent the full spectrum of sperm morphology in investigated South African anurans and Table 2 represents detailed measurements of these sperm. Representative examples of anurans which exhibit the aquatic mode of fertilization will first be described.

The simplest sperm is that of *Rana fuscigula* which has a straight, short symmetrical head, a short midpiece and a single flagellum (Fig. 2). The acrosomal cap is short. The sperm of *Semnodactylus wealii* have similar features except that the flagellum has in addition an undulating membrane and axial rod (Fig. 3). *Xenopus laevis* has a single flagellum but the head is partly corkscrew shaped with one and a half coils (Fig. 4). *Xenopus laevis* sperm furthermore differ from most anurans in that the first few rows of mitochondria surround the posterior part of the nucleus (Fig. 4, insert). *Bufo rangeri* sperm have spear-shaped heads, distinct but short midpieces and flagella with undulating membranes and axial rods (Fig. 5). The head length of aquatic fertilizers ranges from 10.3 to 20.5 µm.

In contrast the sperm of South African anurans which exhibit the terrestrial mode of fertilization have very long and slender heads (*Hemisus marmoratus*, *Leptopelis flavomaculatus*, *Breviceps gibbosus*, *Arthroleptella lightfooti*) or long heavily coiled heads (*Chiromantis xerampelina*) varying from 21.4 to 45 µm in length (Figs 6 & 8) (Table 2). Both the head lengths as well as the acrosome lengths of the terrestrial fertilizers were significantly ($p<0.05$) longer than those of the aquatic fertilizers. However, the flagella of the TF represent either a single axoneme or an axoneme with an undulating membrane and axial rod like the AF. The exception was *Chiromantis xerampelina* which has a single flagellar complex containing two axonemes surrounded by a multitude of microtubules. The term ect-terrasperm is suggested as a new terminology for amphibians which exhibit the terrestrial mode of fertilization. A clear distinction between ect-aquasperm and ect-terrasperm is therefore evident.

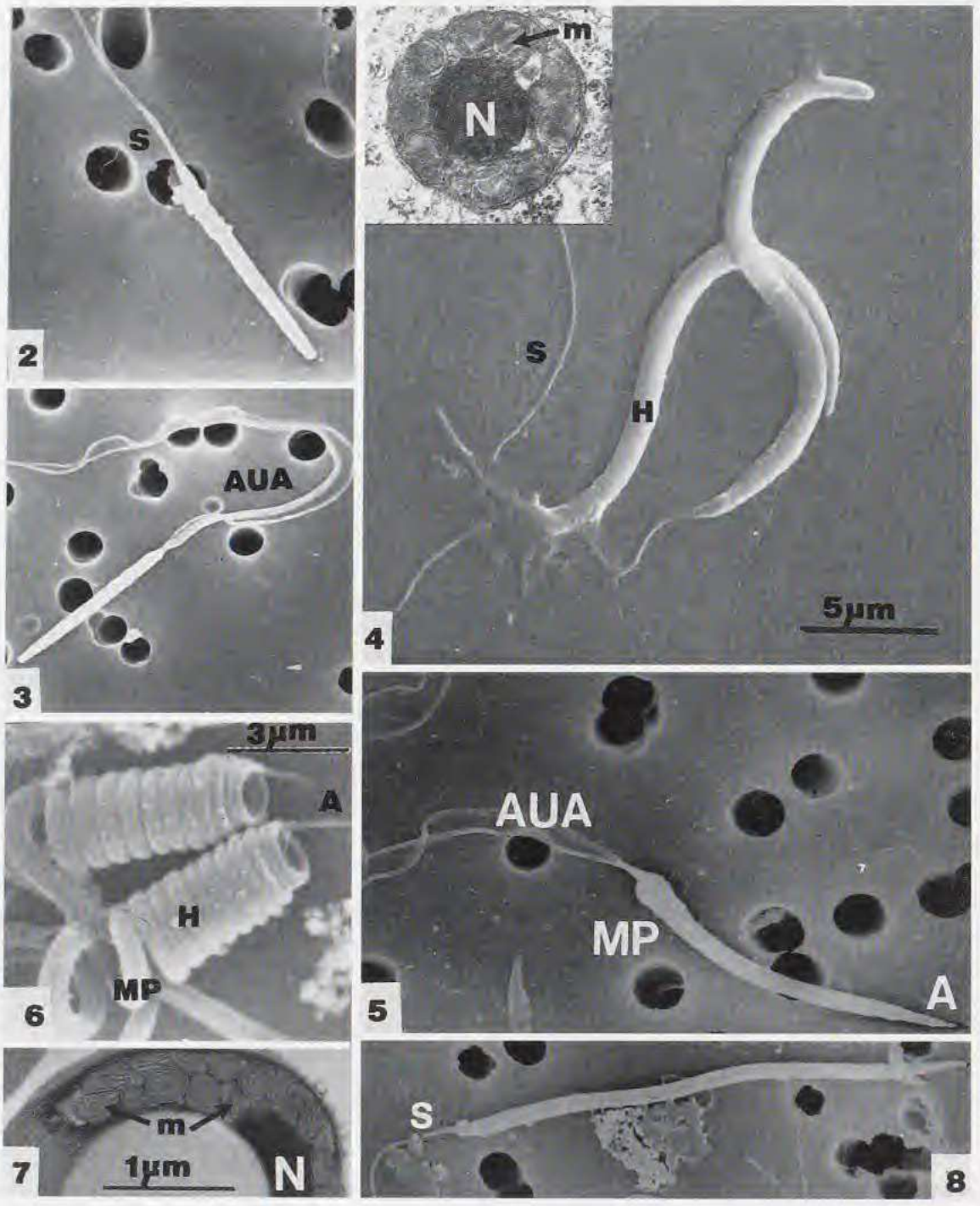
In *Chiromantis* the first few rows of mitochondria also surround the posterior part of the nucleus as in *Xenopus*. Furthermore, *Chiromantis* sperm has spherical mitochondria (Fig. 7) in contrast to *Rhacophorus arboreus* sperm which has elongate mitochondria.

Urodeles and caecilians

Sperm structure in the urodeles and caecilians (limited information) shows less variation than the anurans in terms of basic design. The sperm heads in both these groups are long and slender. While considerable variation exists in the size and form of the flagellum, the basic pattern of axoneme-undulating membrane-axial rod (AUA) seems to occur in all urodele and caecilian species. Their acrosomes are similar in that they extend from the anterior part of the nucleus but do not form a cap that largely overlaps with the nucleus as in anurans. The detailed structure of the acrosome of urodeles and caecilians are also complex and mostly contain an acrosomal rod surrounded by less dense but granular material. All urodele sperm furthermore contain a marginal filament or juxta-axonemal fibre at doublet 8 next to the axoneme, which is absent in caecilians and anurans.

Sperm motility of South African anurans

Sperm of all aquatic fertilizers exhibited forward progression and the VCL varied from 20 to 31 µm/s, VSL from 8 to 23 µm/s, LIN from 40 to 72%, BCF from 3 to 6Hz and ALH from 4.7 to 7.5µm. In contrast the sperm of two species of terrestrial fertilizers (*Breviceps* and *Arthroleptella*) were immotile in a wide range of physiological media and osmotic concentrations varying from 10 to 300 mOsm/kg. In *Chiromantis xerampelina*, sperm only exhibited an initial rapid starspin movement followed by uncoiling of the head and became immotile within a



FIGS 2-8. — Scanning electron micrographs of amphibian spermatozoa. **2:** *Rana fuscigula*. **3:** *Semnodactylus wealii*. **4:** *Xenopus laevis* (inset: transmission electron micrograph of midpiece). **5:** *Bufo rangeri*. **6:** *Chiromantis xerampelina*. **7:** *Chiromantis xerampelina*, transmission electron micrograph of midpiece. **8:** *Breviceps gibbosus*. A, acrosome; AUA, Axoneme-undulating membrane-axial rod; H, Head; m, mitochondria; MP, midpiece; N, Nucleus. S, single flagellum only with axoneme. Holes in membrane filters (Figs 2, 3, 5 & 8) are 3 µm in diameter.

minute. Star symbol plots were used to visually express sperm motility as a pattern of movement (Fig. 9). Distinct motility patterns could be constructed that were clearly species specific. Sperm with partly coiled heads such as *Xenopus* and *Strongylopus* swim in a corkscrew fashion and the linearity of the sperm is almost similar (41.3% and 42.4% respectively) whereas *Bufo* sperm have straight heads and a LIN of 67.6%. Furthermore, more closely related species (*S. grayii* and *P. adspersus*) had distinct sperm motility patterns but also shared many similarities for several motion parameters. This can also be seen in Fig. 9, indicating the similarity in star symbol plots.

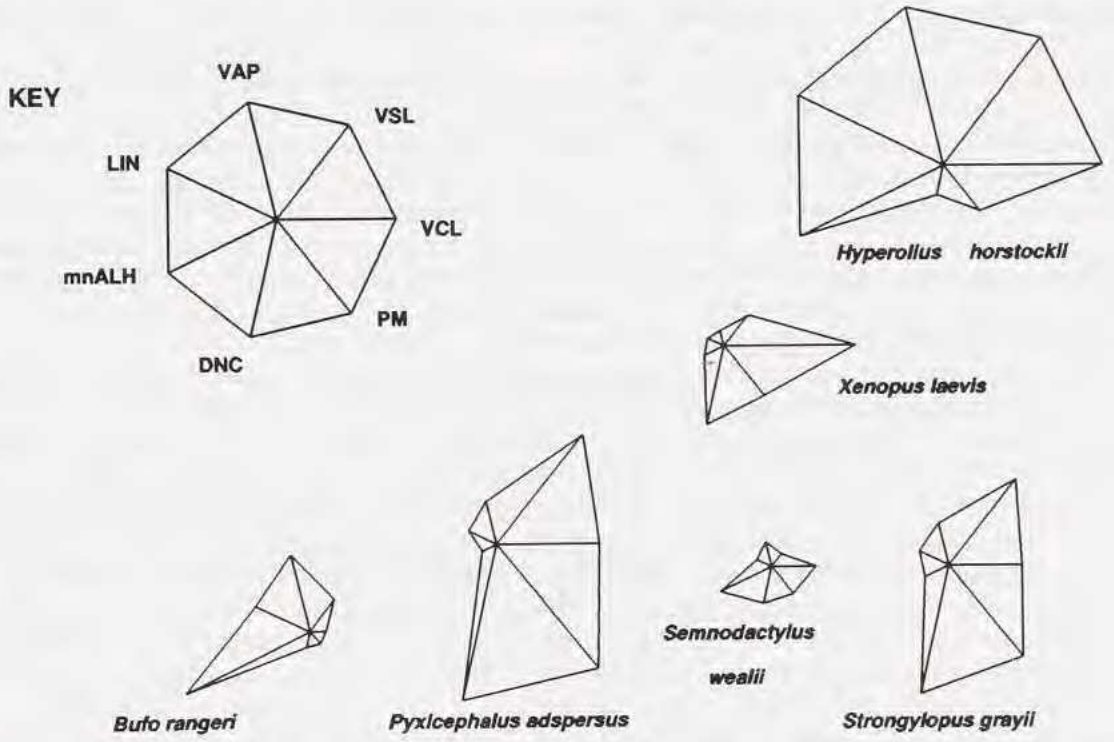


FIG. 9. — Star Symbol Plots (seven motion parameters used - see abbreviations elsewhere in text - PM = percent motile sperm) depicting patterns of sperm motion among six anuran species. For any set of stars (above) the smallest observed value for a parameter is plotted as an arm one-tenth the length of the arm representing the largest observed value.

TABLE 2. — Sperm dimensions of aquatic and terrestrial South African anuran fertilizers.

Fertilization grouping	Head length (µm)	Head width (µm)	Acrosome length (µm)
Aquatic			
mean	16.0	0.7	2.2
(± SD)	(2.2)	(0.4)	(1.2)
Range	10.3 - 20.5	0.1 - 1.6	0.7 - 4.6
Terrestrial			
mean	29.6	0.6	5.9
(± SD)	(6.0)	(0.3)	(1.6)
Range	21.4 - 45.0	0.1 - 1.1	3.2 - 7.3

DISCUSSION

It has been hypothesized that primitive lissamphibians were internal fertilizing and that external fertilization was a secondary reversion in the anurans [10]. The 9+2 axoneme-undulating membrane-axial rod is typically associated with this pattern as well as distinct acrosomal and neck piece features. In reptiles, birds and mammals, where internal fertilization is the rule, a flagellum is associated with a 9+9+2 pattern. There, the nine outer coarse fibres seem to function as a strengthening device for sperm swimming in a viscous environment. The juxta-axonemal fibre as well as the axial rod in amphibians seem to be homologous to the coarse outer fibres 8 and 3 of amniotes and insects [14]. One interpretation may be that the AUA of the early internal fertilizing amphibians represent an intermediate structure from external to internal fertilization and this has been retained as a primitive feature in reversion to external fertilization in many anurans. Alternatively, the AUA of early internal fertilizing Lissamphibia may already represent a simplification of an ancestor that had a 9+9+2 flagellar arrangement. Our data agrees with the interpretation that this feature (AUA) in anurans reflects a plesiomorphic character within a particular group or family [7, 10]. A comparison of three species of *Heleophryne* furthermore supports this view [23]. The flagellar arrangement of particularly the Anura is therefore one of the most important features in determining the relative phylogenetic position of a species. Our results confirm a change from the AUA to a flagellum without an axial rod when examining the more primitive to the more advanced South African anurans.

Our results furthermore show a distinct relationship between sperm head length and mode of fertilization among external fertilizing anurans. The sperm heads and acrosomes of aquatic fertilizing anurans are significantly shorter than those of the TF. It is also known that the egg coverings of TF are generally thicker than those of AF, presumably to protect the eggs against dehydration [13]. A longer sperm head and a larger acrosome which may contain more digestive enzymes may have been an advantage in penetrating these thick egg coats [24]. The information on head and acrosome length may also be of predictive value in other anuran species in establishing whether they belong to the TF or AF grouping. Three South African *Heleophryne* species posses sperm heads with lengths varying from 23 to 28 μm [23]. They are predominantly TF and further support our results. It is therefore clear from our data on South African anurans that sperm head and acrosome dimensions reflect on the fertilization biology rather than the phylogeny and is independent of the type of flagellum (only axoneme or with undulating membrane and axial rod).

The sperm of the three orders of Lissamphibia can be distinguished on the basis of distinct sperm features. A diagnostic feature for urodele and caecilian sperm is a complex acrosome that predominantly extends from the anterior part of the head and does not form a cap surrounding the anterior tip of the nucleus as in all anurans and an acrosomal cap is therefore a diagnostic feature for anurans. Here *Ascaphus* [10] seems to occupy an intermediate position in having a complex acrosome like the urodeles and caecilians but it also forms a cap around the anterior tip of the nucleus. A juxta-axonemal fibre at position 8 is a diagnostic feature for urodeles and separates them from the caecilians and anurans. Anurans on the other hand have developed a minor juxta-axonemal fibre at position 3 which is not clearly defined in urodeles and apparently absent in caecilian sperm [22]. Glycogen packets have furthermore been observed in two ranid species [15] and in *Xenopus* [19] and while the occurrence of these packets is uncommon, they seem to be specific for anurans. *Chiromantis* sperm have spherical mitochondria in contrast to *Rhacophorus* sperm which have elongate mitochondria. The spherical mitochondrion appears to represent the primitive condition in anurans [10] and this may suggest a plesiomorphic character for *Chiromantis* in relation to *Rhacophorus*.

Further sperm structural features that seem to reflect on the fertilization biology of urodeles and caecilians will be briefly discussed. A correlation appears to exist between the length of the neck-piece and the length of time that the sperm is retained in the female cloaca. In

Cryptobranchus external fertilization is the rule and the neck-piece is very small whereas in *Diemictylus*, where sperm are retained in the cloaca for several months, the neck-piece is long [3]. In two species of viviparous Typhlonectidae the acrosome is barbed or hooked [6, 22]. However, in four oviparous taxa of caecilians the acrosomes are spatulate [16, 17, 18]. It appears that the shape of the acrosome of caecilians may be predictive in determining oviparity or viviparity of a given species [22].

Our results on quantitative sperm motility in anurans indicate that sperm from all AF possess motile sperm that swim progressively forward in a physiological medium of 30 mOsm/kg. In contrast sperm from TF are immotile in a wide range of physiological and culture media ranging from 10 to 300 mOsm/kg. In AF, sperm swim in a low osmolality environment even if the male and female cloacae are in close proximity such as in *Xenopus*. Sperm of *Chiromantis xerampelina* (TF) only exhibited a brief spurt (seconds) of hyperactivated motility. In TF the sperm are deposited directly on the eggs and the need for vigorous and longer term motility seems less than in the AF group. It should also be conceded that sperm activation in TF may be dependant on specific substances associated with the egg coat/surface and may explain the immotile status of TF sperm in our experiments.

Finally sperm motion is highly species specific among the South African anurans. The pattern of sperm motion appears to be related to the form of the sperm head, the type of tail (presence or absence of undulating membrane) and the type of flagellar beat. The AF anurans with only an axoneme seem to exhibit greater values for most motion parameters than those with an axoneme-undulating membrane-axial rod and the AF accordingly have larger star symbol plots (Fig. 9).

In summary it appears that several sperm structural features in the Lissamphibia are diagnostic in separating the three main orders, families (urodeles), and even closely related species (*Heleophryne*). It furthermore assists in phylogenetic inferences and is predictive in terms of fertilization biology. Quantitative sperm motility analysis of representative examples of seven of the nine South African anuran families suggest that sperm motility is species specific and also relates to fertilization biology.

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Evolution of Tetrapod Spermatozoa with Particular Reference to Amniotes

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ABSTRACT

Synapomorphies of tetrapod sperm appear to be: nuclear 'shoulders'; elongation, relative to dipnoans, of two longitudinal elements (dense fibres) peripheral to the axoneme adjacent to doublets 3 and 8; and, questionably, development of an annulus. A lissamphibian synapomorphy relative to *Neoceratodus* may have been loss of one undulating membrane, leaving a single undulating membrane adjacent to the fibre of doublet 3. Amniote synapomorphies (retained in Chelonia and Sphenodontida) include: elongation of the distal centriole through the entire length of the moderately elongate midpiece; subspheroidal mitochondria, with concentric cristae; a fibrous sheath; nine peripheral axonemal fibres; inward projections (longitudinal columns) of the fibrous sheath aligned with fibres 3 and 8; loss or transformation of the retronuclear body, present in dipnoans and (as the neck structure) urodeles. A possible crocodilian synapomorphy is a thick dense sheath around the singlets of the axoneme or the distal centriole. Synapomorphies of birds are loss of the subacrosomal cone and, less certainly derived, adhesion of all nine dense fibres to their axonemal doublets (also in monotremes). The conical acrosome, fibrous sheath, and elongate centriole of ratites are symplesiomorphies not proving monophyly. Restriction of the endonuclear canal to the anterior region of the nucleus in other non-passerines and passerines may be a synapomorphy of these, homoplasic with crocodiles and derived ratites (emu). Squamate synapomorphies are: loss of endonuclear canals with restriction of the perforatorial rod to a prenuclear location; intermitochondrial bodies; forward extension of the fibrous sheath into the midpiece; a paracrystalline subacrosomal cone; and, homoplasically, shortening of the centriole. Mammal sperm are distinguished by loss of the perforatorium (and canal), homoplasic with some non-ratite birds, great reduction of the centrioles, and, in therians, (apomorphic?) detachment of peripheral fibres, except sometimes 3 and 8, from the doublets.

RÉSUMÉ

Évolution des spermatozoïdes des Tétrapodes, en particulier des Amniotes

Les synapomorphies des spermatozoïdes des tétrapodes semblent être: des "épaules' nucléaires; l'élongation, en comparaison des Dipneustes, de deux éléments longitudinaux (fibres denses) en périphérie de l'axonème et adjacents aux doublets 3 et 8; et de manière incertaine, le développement de l'annulus. Une synapomorphie des Lissamphibiens en comparaison de *Neoceratodus* peut être la perte d'une membrane ondulante, ce qui laisse une seule membrane ondulante adjacente à la fibre du doublet 3. Les synapomorphies des Amniotes (conservées chez les Chelonia et les Sphenodontia) comprennent: l'élongation du centriole distal sur toute la longueur de la pièce intermédiaire, qui est modérément allongée; des mitochondries subsphériques, avec des crêtes concentriques; une gaine fibreuse; neuf fibres axonémales périphériques; des projections vers l'intérieur (colonnes longitudinales) de la gaine fibreuse alignées avec les fibres 3 et 8; la perte ou la transformation du corps rétronucléaire, présent chez les Dipneustes et (comme structure du cou) chez les Urodèles. Une synapomorphie possible des Crocodiliens est la gaine dense épaisse entourant les singulets de l'axonème ou le centriole distal. Les synapomorphies des Oiseaux sont la perte du cône subacrosomien et l'adhésion des neuf fibres denses à leurs

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JAMIESON, B. G. M., 1995. — Evolution of tetrapod spermatozoa with particular reference to amniotes. *In*: JAMIESON, B. G. M., AUSIO, J., & JUSTINE, J.-L. (eds), Advances in Spermatozoal Phylogeny and Taxonomy. *Mém. Mus. natn. Hist. nat.*, **166** : 343-358. Paris ISBN : 2-85653-225-X.

doublets respectifs. Cette dernière structure existe aussi chez les Monotrèmes et son caractère évolué est moins assuré. L'acrosome conique, la gaine fibreuse et le centriole allongé des Ratites sont des symplésiomorphies qui ne prouvent pas leur monophylie. La restriction du canal endonucléaire à la région antérieure du noyau chez les autres non-passereaux et chez les Passereaux peut être une synapomorphie pour ces groupes, homoplasique avec les Crocodiles et les ratites évolués (Ému). Les synapomorphies des Squamates sont: la perte des canaux endonucléaires avec la restriction de la baguette du perforatorium à une position pré-nuclaire; des corps intermitochondriaux; une extension vers l'avant de la gaine fibreuse dans la pièce intermédiaire; un cône subacrosomien paracristallin; et de manière homoplasique, le raccourcissement du centriole. Les spermatozoïdes des Mammifères se distinguent par la perte du perforatorium (et du canal) qui est homoplasique avec certain Oiseaux non-ratites, la grande réduction des centrioles, et, chez les Thériens (apomorphie?), le détachement des fibres périphériques, exceptées parfois les 3 et 8, des doublets.

This chapter constitutes a brief review, with new material, of the sperm of the amniotes as a whole and attempts to reconstruct the features of the spermatozoon of the ancestral Amniota and the course of spermatozoal evolution, as indicated by deduced synapomorphies, in the amniote classes. This reconstruction is based on intuitive consideration, and also on a cladistic analysis [22], of spermatozoal ultrastructure in the constituent tetrapod groups. Further reviews of the ultrastructure of vertebrate sperm may be found in the recent publications of JAMIESON [21] and HEALY & JAMIESON [16, 22]. Material of dipnoan and urodele sperm is included for comparative purposes.

MATERIALS AND METHODS

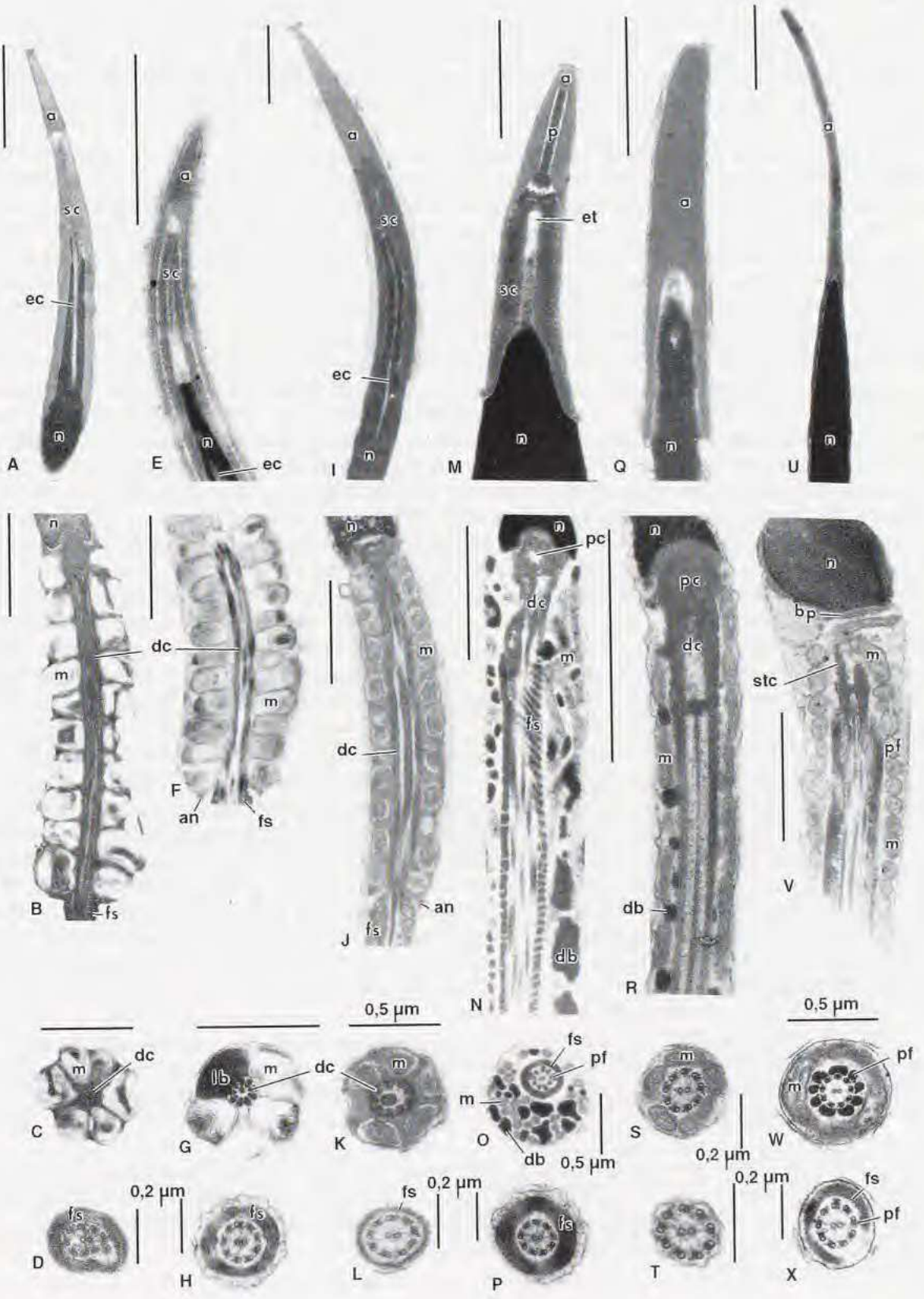
Testes and ducts were dissected from euthanased specimen(s) of *Neoceratodus forsteri* (Dipnoi), *Taricha granulosa* (Urodela), *Emydura kreffti* (Chelonia), *Sphenodon punctatus* (Sphenodontida), *Crocodylus johnstoni* (Crocodilia), *Lampropholis delicata* (Scincidae, Squamata), *Geopelia striata* (Columbiformes, Aves) and *Rhinolophus megaphylla* (Chiroptera, Mammalia). Processing of the tissues was as in [27].

RESULTS AND DISCUSSION

Comparative ultrastructure of amniote spermatozoa

The acrosome, endonuclear canals and perforatoria. All amniote classes (Reptilia, Birds and Mammals) contain some species, or a majority of species, in which the acrosome has a plesiomorphic tripartite structure which, from the evidence of its presence in all three lissamphibian orders (Urodela, Gymnophiona and Anura) [25], was already present in early tetrapods ancestral to Lissamphibia and Amniota. In the plesiomorphic tripartite acrosome the acrosome vesicle forms an elongate, narrow cone symmetrically located on the tip of the nucleus which narrows within it to a point. The acrosome vesicle encloses a similarly shaped subacrosomal cone and axially within this there are one to three rodlike perforatoria. The pointed form of the acrosome, presence of the subacrosomal cone, and tapering of the tip (rostrum) of a

FIG. 1. — Examples of sperm ultrastructure in selected amniote classes and orders. The species depicted in each vertical column are **1:** *Emydura kreffti* (Chelonia); **2:** *Sphenodon punctatus* (Sphenodontida); **3:** *Crocodylus johnstoni* (Crocodilia); **4:** *Lampropholis delicata* (Scincidae, Squamata). **5:** *Geopelia striata* (Columbiformes, Aves); and **6:** *Rhinolophus megaphylla* (Chiroptera, Mammalia). The first transverse row, A, E, I, M, Q, and U, are longitudinal sections through the sperm head (acrosome and anterior nucleus). The second row, B, F, J, N, R, and V, are longitudinal sections through the midpiece and, in all except F, the posterior region of the nucleus. The third row, C, G, K, O, S, W, are transverse sections through the midpiece. The fourth row, D, H, L, P, T and X, are transverse sections through the principal piece. Abbreviations: a, acrosome vesicle; an, annulus; bp, basal plate; db, dense bodies (mitochondrial transformations); dc, distal centriole; ec, endonuclear canal; et, electron-lucent epinuclear region; fs, fibrous sheath; lb, lateral body at anterior region of distal centriole; m, mitochondria; n, nucleus; p, putative perforatorium; pc, proximal centriole; pf, peripheral dense (coarse) fibres; sc, subacrosomal cone; stc, striated column. Scale bar = 1 µm unless otherwise indicated.



cylindroid nucleus within this, are seen in the Chelonia [13, 16, 19, 22] (Fig. 1A), Crocodilia (*Caiman crocodylus* [45], and *Crocodylus johnstoni*, Fig. 1I), Sphenodontida (*Sphenodon*) [16, 22] (Fig. 1E), Squamata [5, 6, 13, 27] (Fig. 1M), non-passerine birds [24, 37] (Fig. 1Q), and monotremes [7]. All of these plesiomorphic representatives of their classes, with the exception of mammals, and, in the non-passerines, the columbiforms (Fig. 1Q), possess the perforatorial rod or rods.

In the Squamata the subacrosomal cone has a paracrystalline substructure [5, 6, 13], recently confirmed for Sphenomorphus and Eugongylus group skinks, the gekkonid *Heteronotia binoei*, and in snakes [35]. It constitutes a basal synapomorphy of the Squamata [22].

The perforatorial rod in the Squamata is wholly prenuclear (Fig. 1M) but this restriction is clearly apomorphic. The plesiomorphic condition is seen in basal lissamphibians where a rod penetrates the nucleus to varying depths, each within an endonuclear canal: *viz.* urodeles [41] and the primitive anurans *Ascaphus* [25], *Discoglossus* (spermatid only) [47, 48], and bombinids, including *Bombina* and *Alytes* [43]. The number of endonuclear canals and of enclosed perforatoria is one in basal Lissamphibia, in the caiman (though poorly substantiated by micrographs), tinamou, rhea and non-passerines (e.g. galliforms), but in the Chelonia and *Crocodylus johnstoni* there are two or three canals and there are two in *Sphenodon*. There are three endonuclear canals in the sperm of the sturgeon, *Acipenser sturio*. Although there appears to be only a single canal in the coelacanth, *Latimeria chalumnae*, this contains two or three perforatoria [21] and two to four perforatoria are seen in *Neoceratodus* (Fig. 3B). It is therefore probable that the presumed common ancestor of Amphibia and amniotes possessed more than one perforatorium and endonuclear canal. A single canal appears basic to all amniotes above turtles and *Sphenodon* [22]. In *Acipenser* the canals are spiralled around each other as they are in turtles, *Sphenodon* and *Crocodylus johnstoni*. The spiral arrangement or at least the presence of one or more endonuclear canals may well be a synapomorphy for the Osteichythes, a monophyletic clade including the Actinopterygii (Ray-finned fish), Sarcopterygii and, within the latter, the Tetrapoda. That a canal was present even earlier is evidenced by presence of a canal and filamentous perforatorium in lamprey sperm. The canals are absent (presumed lost) in the highly simplified sperm of the Chondrostei and Neopterygii [21].

In birds, a conical acrosome vesicle penetrated almost to its tip by a subacrosomal space which contains a rodlike perforatorium has been demonstrated ultrastructurally in the non-passerines turkey, *Meleagris gallopavo*, chicken, *Gallus domesticus*, guinea fowl, *Numida meleagris* (e.g. [55]), the mallard duck, *Anas platyrhynchos* [20], and the quail *Coturnix coturnix* [20], parrots [24] and in the ratites (palaeognaths) tinamou, *Eudromia elegans* [1], ostrich, *Struthio camelus* [3, 51, 52] and emu, *Dromaius novaehollandiae* [3]. Like the sperm of ratites and other birds, parrot sperm differ from those of reptiles in reduction of the subacrosomal material (subacrosomal cone, excluding any perforatorium) to a negligible amount [24]. In the columbiforms even a perforatorium is absent although, at least in *Geopelia striata* (Fig. 1Q), some longitudinally orientated subacrosomal material is present and lacunae are present in the nucleus which may represent a vestigial endonuclear canal.

Not all non-passerines possess a conical acrosome. A small, approximately spherical acrosome has been described for the white-naped crane, *Grus vipio* [40], for *Jacana jacana* [46] and most Charadriiformes [11], and for the wood pecker *Melanerpes carolinus* ([18]. These latter avian taxa are considered to be advanced non-passerines, on the basis of DNA hybridization studies [49, 50].

In the non-passerine and suboscine spermatozoon the acrosome is short relative to the nucleus, as in reptiles [22, 27, 28], in contrast to the oscine spermatozoon which has an extremely large acrosomal complex. In passerines the acrosome vesicle becomes an elongate single-keeled helix, with no evident subacrosomal cone, in, for instance, finches [30, and KOEHLER, this volume].

The endonuclear canal extends almost to the base of the nucleus in Chelonia and *Sphenodon* [16, 17, 22], and this is considered a plesiomorphic state [22]. This condition also occurs in putatively more primitive ratites (i.e. tinamou [1]), where it is probably also plesiomorphic relative to the shorter condition in other non-passerines and in advanced ratites, being wholly prenuclear in the emu [3]. However, there is a possibility that deep penetration in ratites is secondary [22]. The canals extend deeply into the nucleus in *Crocodylus johnstoni* (Fig. 1I). A single canal is depicted as penetrating only the nuclear rostrum in the caiman [45] but the extent requires further investigation. The endonuclear canal is limited to the anterior 1 to 2 μm of the nucleus in rooster, guinea fowl, turkey and parrots and the anterior third of the nucleus in the ostrich [3]. The canal is lost in mammals and, homoplasically, in squamates.

In mammals, as in the bat *Rhinolophus megaphylla* (Fig. 1U), rodlike perforatoria do not occur. Although monotremes [7], retain the elongate conical structure of the acrosome and the subacrosomal cone, this form is greatly modified in the Eutheria (e.g. [44]) and Metatheria [53]. In Eutheria the acrosome is flattened, with the notable exception of the pangolin [33].

The nucleus and nuclear fossa. The nucleus is plesiomorphically elongate in amniotes from Chelonia through *Sphenodon*, crocodiles, squamates, birds, monotremes and, in therian mammals, the pangolin alone [33], as in lissamphibians.

Representation of the basal nuclear fossa, loosely termed the implantation fossa, is variable in amniotes but some of this variation may be spurious as it is difficult to establish the shape of the fossa. It appears poorly developed in the sperm of *Caiman crocodylus* [45] but has a low dome-shaped form in *Crocodylus johnstoni* (Fig. 1J). It is small and compact in turtles, tuatara, rooster, guinea fowl, and squamates excepting some skinks in which it is narrowly funnel-shaped. In the ratites it has a a triple profile (references in [22]). It is dome-shaped to rounded conical in eugongyloid skinks [28]. It is a shallow cone in the gekkonid *Heteronotia binoei* and compactly conical in the pygopodid *Lialis burtoni* [26]. The compact form appears to be plesiomorphic for amniotes [22].

The annulus. A dense ring, the annulus, at the posterior end of the midpiece is a feature of many metazoan sperm. It is clearly plesiomorphic for amniotes, occurring in all classes [22] but absence in Dipnoi possibly indicates apomorphic re-acquisition in tetrapods. It is well developed in Chelonia, *Sphenodon* [16, 17, 22], *Caiman crocodylus* [45], the American Alligator [39] and *Crocodylus johnstoni* (Fig. 1J). In squamates, it has been demonstrated for *Lacerta vivipara* [8], *Cnemidophorus sexlineatus* [34], sphenomorph and eugongyloid skinks [27, 28], *Heteronotia binoei*, *Varanus gouldii flavirufus* [35], and, though reduced in some species, in snakes [23, 35]. The annulus is basic to birds, being seen in ratites, rooster, guinea fowl, and columbiforms [2] but is apomorphically absent in parrots [24]. It is weakly developed in monotremes [7]. Two structural categories of annulus have been recognized in therian mammalian sperm, based on the profile of the annulus as viewed in longitudinal section: triangular (e.g. bats, dormouse, Chinese hamster, antelope) and semicircular (e.g. mouse, guinea pig, chinchilla, ram) [12]. However, the taxonomic and phylogenetic significance of the shape of the annulus is questionable, given that in the Rodentia both categories are encountered.

The number of mitochondria. The number of mitochondria seen in transverse section of the midpiece is very variable in amniotes but some of the apparent variation requires confirmation, particularly as there is variation along the midpiece. Determination of the total number by scanning electron microscopy would be desirable. It appears that a number, in transverse section, in the order of 6 to 9 may have been plesiomorphic; 6 has been recorded for the Chelonia, 9 in *Sphenodon*, 6 to 8 in *Caiman crocodylus* [22, 45] and 6 in *Crocodylus johnstoni* (Fig. 1K). In the remaining amniotes, a trend towards reduction, in transverse section, to 4 in birds and monotremes has been suggested [22]. It is 4 in ratites (e.g. [51]) and in the turkey [54] and in the order of 5 in *Geopelia striata* (Fig. 1S). However, in squamates the number remained plesiomorphically high in lizards or showed apomorphic increase to as many as 14, in snakes,

while a reduction to 2 in gekkos was correlated with intrusion of intermitochondrial material of supposed mitochondrial origin into the transverse section of the midpiece. Further variability in numbers is now known though it cannot be fully documented here. For instance, large numbers of small mitochondria occur, it seems apomorphically, in eugongyloid skinks [28] and approximately 9 have been observed in transverse section of the budgerigar sperm [24]. The mitochondria are subspheroidal in Chelonia, *Sphenodon* and crocodiles and this may reasonably be inferred as the plesiomorphic condition. The number of tiers of mitochondria in longitudinal sequence is in the order of 10 in turtles [16] which is also presumed to be plesiomorphic. In the spiral midpiece of mammals, the number of gyres varies from 55 to 300 [10] but is not specified for monotremes [7].

Structure of the mitochondria. In turtles (Fig. 1B, C), tuatara (Fig. 1F, G), *Caiman crocodylus* and *Crocodylus johnstoni* (Fig. 1J, K), the mitochondria have a form known only in the sperm of one other amniote, the Woolly opossum, *Caluromys philander* (see [36]; [9]). The mitochondrial cristae in these three taxa are concentric and usually surround a large central dense body. In all other amniotes studied, the cristae have a "conventional" appearance, being linear or curved, as in Lissamphibia, but never concentric, and do not surround a dense body.

Linear cristae in spermatozoal, as in somatic mitochondria, must be accepted as a plesiomorphic condition for tetrapods as they are normal for metazoan sperm, including fish. The concentric arrangement with dense body appears to be an apomorphy acquired early or initially in amniote evolution and retained paraphyletically in the tuatara, crocodile, and turtle clades [16, 22]. In spermatids of *Caiman crocodylus* [45] and in at least some mitochondria of spermatids of *Sphenodon* [16, 22], the cristae have the linear appearance usual for metazoan sperm and the concentric arrangement is a late development. Phylogenetic "reversion" of mitochondrial of concentric cristae to the linear condition seen in other amniotes would need only suppression of this final transformation [22]. Presence of concentric cristae and the intramitochondrial body in the woolly opossum is construed as homoplasic although the possibility that ancestral mammals retained this condition from basal amniotes cannot be ruled out [16].

The intermitochondrial rings or dense bodies of squamate sperm are regarded as derivations of the intramitochondrial dense bodies [6, 16, 22] and as such a "reminiscence" of the occurrence of concentric cristae in the ancestry of squamates. Origin of intermitochondrial material from mitochondria has been confirmed ontogenetically in the sperm of some squamates [35]. Extramitochondrial dense bodies are almost limited to squamates but are seen, poorly developed, in *Geopelia striata* (Fig. 1R) in which, although appearing homoplasic, they may well indicate persistence of a genetic basis laid down in early amniotes.

The centrioles. Presence of the proximal centriole can be regarded as plesiomorphic for tetrapods and is seen in all amniote classes. It persists, well developed, in monotremes [7], but is absent from mature therian mammals, for instance, the rat [10] and the bat, *Rhinolophus megaphylla* (Fig. 1V).

A distal centriole is at most a vestige in mature mammalian sperm [10] (see also Fig. 1V), but is well developed in sperm of anurans [32], Chelonia (Fig. 1B, C), *Sphenodon* (Fig. 1F, G), crocodiles [16, 22] (Fig. 1J, K), squamates [13] (Fig. 1N), and birds [1, 2] (Fig. 1R). The distal centriole, forming the basal body of the axoneme, is plesiomorphically short in vertebrates, including the Lissamphibia and squamates [27]. In contrast, the distal centriole extends the entire length of the long midpiece in turtles (Fig. 1B, C), the tuatara (Fig. 1F, G), crocodiles (Fig. 1J, K), and ratites, an apparent basal synapomorphy of amniotes. These elongate centrioles differ from most metazoan basal bodies in being penetrated by two central singlets from the axoneme. Thus in spermatids of the ratite *Rhea*, the distal centriole elongates and, late in spermiogenesis, becomes penetrated by a central pair of tubules from the developing axoneme [38]. The shorter, though still elongate distal centriole in the rooster and the somewhat shorter centriole in guinea fowl (0.6 μm) and *Geopelia striata* (0.5 μm) (Fig. 1R), the short centriole in squamates, and the

vestigial centriole in monotremes possibly represent secondary reduction in length of the centriole [16], culminating in almost total reduction in therian mammals.

The distal centriole is embedded in a ring of dense material in all of the amniotes for which it has been investigated. A cross striated dense body lateral to the proximal centriole appears to be a basal synapomorphy of amniotes but its homology across the various groups requires confirmation. It is seen in tuatara, and the caiman where homology with the nine striated columns of eutherian sperm has been suggested [16] but has yet to be demonstrated in *Crocodylus johstoni*. In the skink *Nangura* it is represented by a bilateral laminated structure [27] and shows various manifestations in other squamates. It is does not appear to have been reported in birds.

The axoneme and appurtenances. An annulated, helical, dense fibrous sheath (Fig. 1B, D, F, H, J, L, N-P, X) must, clearly, have developed in the earliest amniotes as it is present in all amniote classes. Homology of this sheath across the different classes is reinforced by the presence in mammals of two spurlike inwardly directed triangular processes, seen in cross section of the sperm, in the vicinity of doublets 3 and 8, where they form large 'longitudinal columns' [10], though at most weakly developed in monotremes, and in ratites. In the non-passerines, rooster and guinea fowl, the fibrous sheath has transformed into an amorphous sheath [55] while in parrots [24] and doves (*Ocyphaps lophotes*, pers. obs., and *Geopelia striata*) it is lost.

In most of the amniotes investigated the fibrous, or amorphous, sheath commences immediately behind the midpiece. This condition is seen in turtles (Fig. 1B), *Caiman crocodylus* and *Crocodylus johnstoni* (Fig. 1J), ratites, non-passerines (absent in parrots and doves) and mammals. However, in squamates the fibrous sheath extends anteriorly well into the midpiece, a clear squamate autapomorphy [16, 22, 27], as in *Lampropholis delicata* (Fig. 1N, O).

An external longitudinal protuberance (rib) on each side of the fibrous sheath is seen in many amniotes, including eutherians, and is particularly well developed in the tinamou [1]. A sheath of putative glycogen external to the fibrous sheath was found to be limited to the tinamou [38] and cannot be ascribed to the plesiomorphic amniote sperm.

Nine longitudinal dense fibres (coarse fibres) peripheral to the nine axonemal doublets, or to the distal centriole also where this is elongated as in *Emydura*, *Sphenodon* and *Crocodylus*, are a fundamental feature of amniote sperm, being found in all classes [22, 27]. The presence of nine fibres is an autapomorphy and simultaneous symplesiomorphy of the amniotes, though nine appear homoplasically in other groups, such as some lampreys and the osteoglossomorph fish *Pantodon* [21], and in heterobranch and cephalopod molluscs [14, 15]. The dense fibres are small in turtles (Fig. 1C), *Caiman crocodylus* and *Crocodylus johnstoni* (Fig. 1K), the tuatara (Fig. 1G), squamates (Fig. 1O), birds and monotremes. They have been observed in turkey, rooster and, though requiring confirmation, in guinea fowl [55], the mallard duck [20], in parrots [24], and in the anteriormost region of the principal piece of ratite spermatozoa [1, 3, 51, 52] but are described as "tiny" for the rhea, are absent from the tinamou [1], and are greatly reduced in columbiforms (Fig. 1S). They are present in suboscine and the more apomorphic oscine passerines, being larger in the latter. They are large and diverse in shape in marsupials above the didelphids, and in eutherian mammals (Fig. 1W). There thus appear to be trends to enlargement of the peripheral fibres in passerines and non-monotreme mammals, with diversification in the latter, and to reduction in ratites, and doves. In Chelonia, *Sphenodon*, *Caiman crocodylus* (but not apparently *Crocodylus johnstoni*) and in squamates, the fibres at doublets 3 and 8 are enlarged [16, 22] and are possibly homologous with the axial fibre, at 3, and juxta-axonemal fibre at 8, in lissamphibian sperm.

Significant synapomorphies are here suggested for amniote groups on the basis of the configuration of the peripheral axonemal fibres. In reptiles the peripheral fibres at 3 and 8 are detached from their corresponding doublets while the other seven fibres are attached to their doublets. In birds and monotremes all of the peripheral fibres are attached to the corresponding doublets [12]. In contrast to both of these assemblages, in therian mammal sperm the peripheral

fibres are detached from their doublets with the exception that fibres 3 and 8 may be close to or attached to their doublets [12, pers. obs.].

The peripheral fibres are usually situated in the midpiece with some extension into the principal piece as in turtles [22], the caiman [45], non-passerines [2], tuatara [22], and monotremes [7]. In the rhea and tinamou dense fibres are present only in the proximal principal piece. Very small dense fibres are present only in the distal region of the midpiece in the rooster and mallard; dense fibres in turtle dove sperm disappear before maturation is complete (see review by ASA & PHILLIPS [2]), though they persist through a short region of the midpiece in *Geopelia striata*. In eutherians and marsupials they extend far into the principal piece. However, in squamates, the only well developed, though small, peripheral fibres at the level of the annulus are the double fibres at doublets 3 and 8 and by the beginning of the principal piece all nine dense fibres are already vestigial or absent [27]. The fibres extend through most of the length of the sperm cell in oscine passerines [38].

In turtles, *Sphenodon* [16], *Crocodylus johnstoni*, and in skinks [13, 27, 28], the nine peripheral dense fibres are partly displaced from the radii of the triplets of the distal centriole into the gaps between adjacent triplets; the fibres are coradial with the doublets in the axoneme. These locations have been regarded as plesiomorphic for amniotes in Fig. 1. Dense material surrounds the central singlets in Chelonia (Fig. 1C), *Sphenodon* (Fig. 1G), and in crocodiles (Fig. 1K). In crocodiles the compact dense sheath appears to be a distinctive synapomorphy.

Snake sperm are characterized, apomorphically, by multilaminar membranes in place of the normal plasma membrane of the midpiece and axoneme [23, 35]; see also pygopodids [26].

The hypothetical plesiomorphic amniote spermatozoon (Fig. 2)

From the above comparative and cladistic considerations of the anatomy of amniote sperm, the following features may be attributed to the hypothetical plesiomorphic amniote spermatozoon. Relative to the tetrapod ground plan, deduced from common features of the amniote and lissamphibian sperm, amniotes are seen to have few basal synapomorphies. These are indicated, among plesiomorphic features of the amniote sperm, below.

The spermatozoon was elongate and filiform, with a hollow anterior conical acrosome vesicle overlying a simple subacrosomal cone. The base of the acrosome invested the tapered anterior tip (rostrum) of the nucleus and rested on pronounced nuclear 'shoulders'. The subacrosomal space within the acrosome contained two or three axial rods (putative perforatoria) or possibly only one rod. These penetrated the nucleus deeply, almost to its base, in endonuclear canals. At the base of the nucleus there was a compact fossa (implantation fossa) with which were associated two triplet centrioles of which the distal formed the basal body of the flagellar axoneme. The distal centriole was extremely elongate (amniote synapomorphy), traversing the entire length of the moderately elongate midpiece, the latter being defined by the presence of mitochondria. The mitochondria were subspheroidal, with concentric cristae and intramitochondrial dense body (amniote synapomorphies), and formed a circlet of several in cross sectional view around the distal centriole and several tiers in longitudinal section. The posterior end of the midpiece was defined by a subplasmalemmal dense annulus possibly homologous with the ring seen in urodele sperm [42]. The axoneme proper, consisting of nine peripheral doublet and two central singlet microtubles, posterior to the midpiece and constituting the principal piece, was surrounded by a fibrous sheath (amniote synapomorphy) which plesiomorphically was annulated. The terminal portion of the axoneme formed a short endpiece defined by the absence of the sheath. The elongate distal centriole and, internal to the fibrous sheath, a long anterior region of the axoneme, was surrounded by nine dense peripheral fibres, one to each triplet or doublet respectively (an amniote synapomorphy relative to fibres at 3 and 8, only, in lissamphibians). In the principal piece, two fibres, at doublets 3 and 8, were aligned with an inward projection

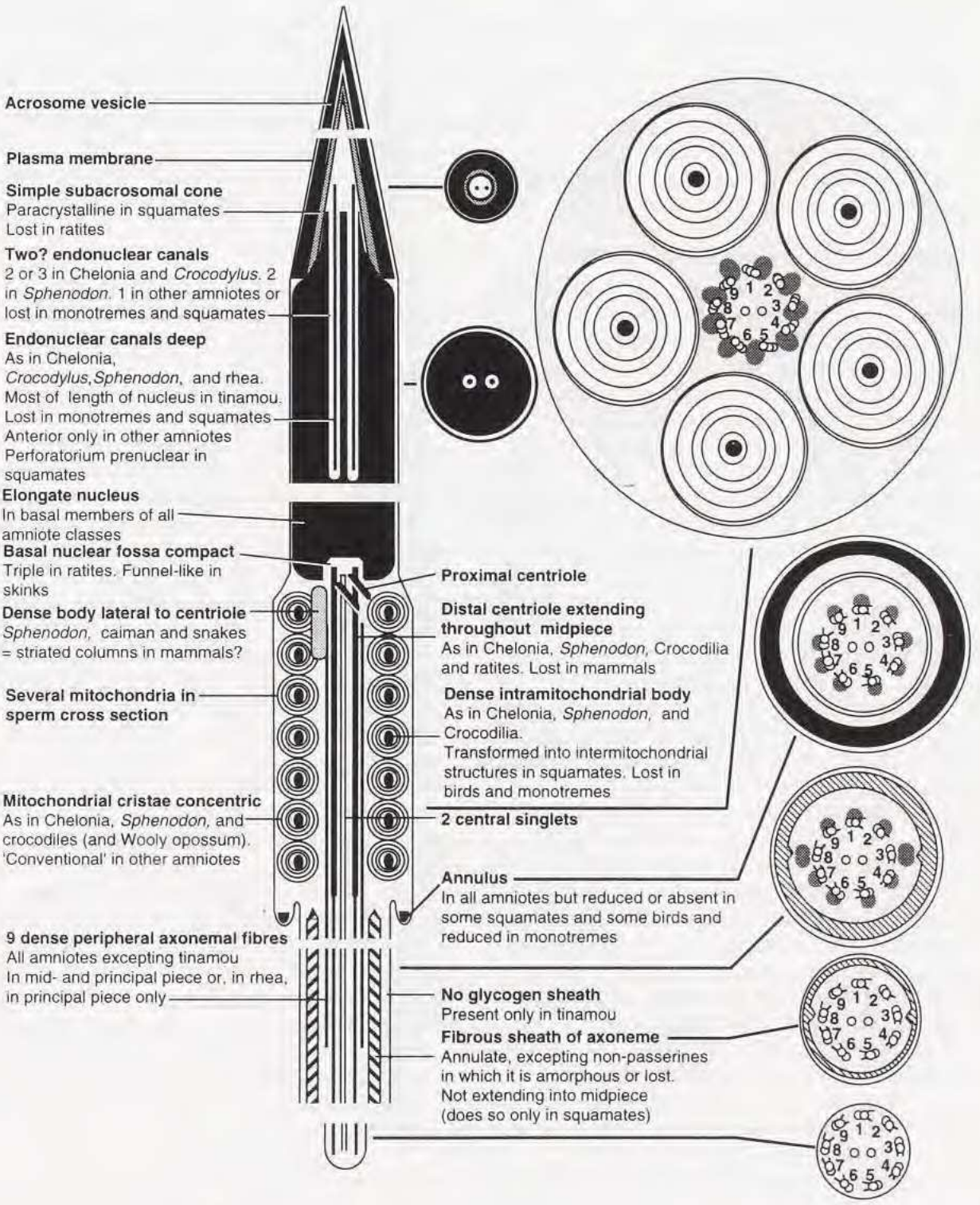


FIG. 2. — Diagrammatic representation of the hypothetical plesiomorphic amniote spermatozoon, the features of which are deduced in the text.

(longitudinal column) of the fibrous sheath. It is possible that the two fibres were enlarged and laterally displaced, and that all fibres in the centriolar region intruded into the inter-triplet radii, as in 'lower' amniotes (Chelonians, *Sphenodon* and crocodiles). As nine peripheral fibres are seen in lampreys and *Pantodon*, it might be considered that nine is the basic tetrapod, rather than merely amniote, number and that amphibians have lost all but those represented by the fibres at doublets 3 and 8 but there is no evidence in extant Lissamphibia for such a reduction and the presence of only two lateral elements in dipnoans and *Latimeria* suggests that nine fibres were an amniote synapomorphy, albeit homoplasic with some fish taxa. The retronuclear body seen in dipnoans and urodeles was lost or possibly transformed into the striated pericentriolar material.

The hypothetical plesiomorphic lissamphibian spermatozoon (Fig. 3)

A survey of the literature, of which only a few references can be given here, together with personal observations, on the sperm of urodeles [42], Gymnophiona [56], and Anura [25, 31, 32, 43] permits the following generalizations as to the ultrastructure of the sperm of ancestral lissamphibians (Fig. 3).

The hypothetical plesiomorphic lissamphibian spermatozoon may be attributed an anterior acrosomal vesicle forming a hollow, cone which overlay a cone of subacrosomal material. This embraced the tapered anterior end (rostrum) of the elongate nucleus. In Anura, basically, the acrosome vesicle and underlying cone embrace the nuclear rostrum. Urodeles (at least salamandrids and plethodontids, pers. obs.) are apomorphic in that the posterior limit of the acrosome vesicle is anterior to the nucleus, only the subacrosomal cone investing the nuclear rostrum. At the posterior end of the acrosome (vesicle and subacrosomal cone) the lissamphibian sperm nucleus plesiomorphically forms characteristic 'shoulders' posterior to which its form is cylindrical. Axially, within the acrosome, there is a rod, the putative perforatorium, which deeply penetrates the nucleus within an endonuclear canal. An axial rod (perforatorium), though lodged posteriorly in a much shorter endonucler canal, is also present in gymnophionids. As it is also present in the primitive frogs *Discoglossus* [43] and *Ascaphus* [25] and in the bombinids *Bombina* and *Alytes* [43], it is reasonable to deduce that a long acrosome rod and endonuclear canal is plesiomorphic for the Lissamphibia. This is endorsed by persistence of this condition in lower amniotes, the Chelonia, Sphenodontida and at least *Crocodylus johnstoni* in the Crocodilia. The base of the nucleus was indented as a basal nuclear fossa which contained the proximal centriole behind which lay the distal centriole which formed the basal body of the axoneme. Behind this, retronuclear material may have persisted to form the neckpiece but not as well developed as that seen in urodeles [42] above the cryptobranchs.

The structure of the sperm tail or flagellum is highly distinctive of the Lissamphibia. The axoneme has the structure usual for eukaryotes of nine peripheral doublet and two central singlet microtubules but it has distinctive appurtenances. To the generalized lissamphibian sperm can be attributed two longitudinal fibres, one on each side of the axoneme, adjacent to doublets 3 and 8. The fibre at doublet 8 is closely adjacent to the ring of doublets and may be termed the juxta-axonemal fibre at 8. This fibre is usually, but secondarily, absent in Anura. The fibre associated with doublet 3 is separated from the axoneme by a thin sheet of cytoplasm, the undulating membrane (with or without intervention of a juxta-axonemal fibre), and is termed the axial (major) fibre. The epithet axial refers to reported undulation of the axoneme around this stiff fibre in the Urodela and some primitive frogs (see KWON & LEE, this volume).

In urodele sperm, the axial fibre, at doublet 3, is connected to the axoneme by the undulating membrane but, typically, as in *Taricha granulosa* (Fig. 4C), there is no intervening juxta-axonemal fibre [4, 42]. *Plethodon albagula* [25, 32] is exceptional, of urodeles studied, in having a density on the adaxonemal end of the undulating membrane but it is questionable, because of its connection to dense bodies near the major fibre rather than to the fibre, that this is

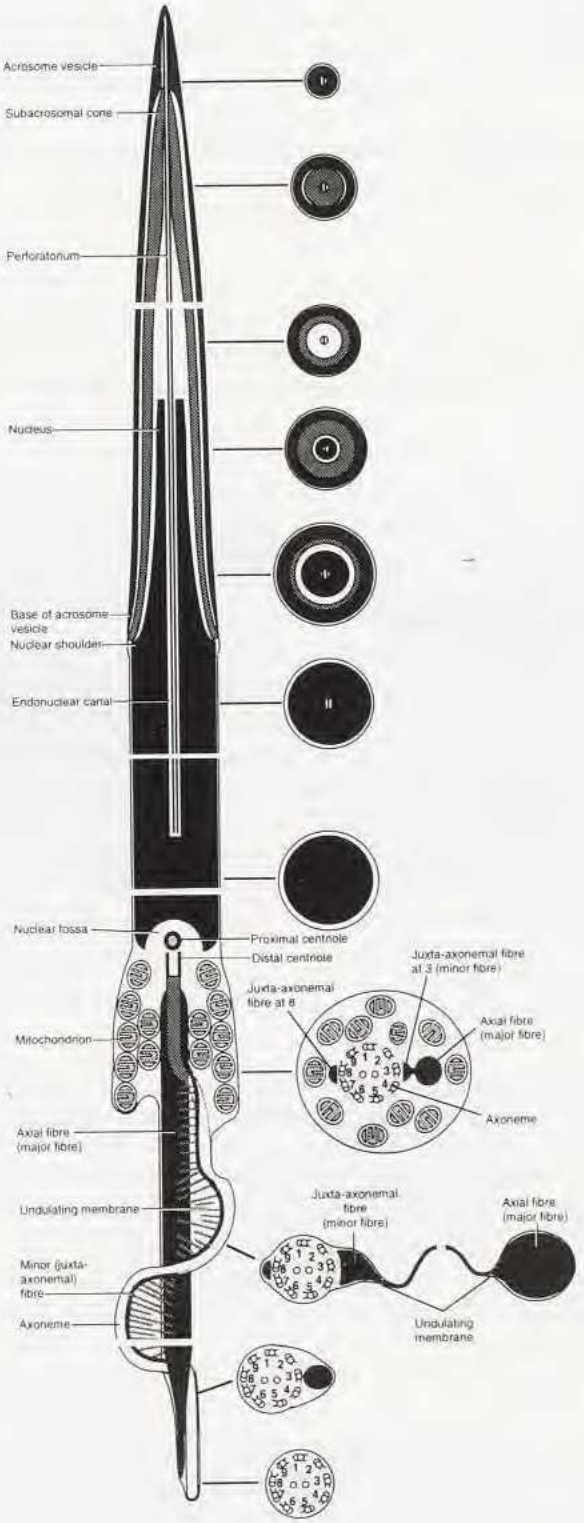


FIG. 3. — Diagrammatic representation of the hypothetical plesiomorphic lissamphibian spermatozoon, the features of which are deduced in the text.

homologous with the anuran juxta-axonemal fibre at 3. An axial fibre with undulating membrane is present in caecilians, in which juxta-axonemal fibres are absent [56]. Presence of a longitudinal fibre, near doublet 3, connecting to the axoneme via an undulating membrane appears, at least on first analysis, to be a synapomorphy of the Lissamphibia irrespective of whether it is subdivided into an axial (major) and juxta-axonemal (minor) fibre or is accompanied by a fibre at doublet 8. The possibility has been mooted [21, 25] (see also next section) that it is the *unilateral* location of the undulating membrane and its axial fibre, rather than presence of undulating membranes *per se*, which constitutes the synapmorphic condition for the Lissamphibia.

Pre-tetrapod presence of undulating membranes

Two longitudinal elements occur, one at each of doublets 3 and 8, in the dipnoan *Neoceratodus forsteri* (Fig. 4A) and the actinistian *Latimeria*. Sarcopterygian fish, and particularly dipnoans, appear to be the nearest extant non-tetrapod relatives of amphibians. Two, bilateral, elements which also occur at doublets 3 and 8 in Chondrichthyes are deduced to have been convergently acquired [21].

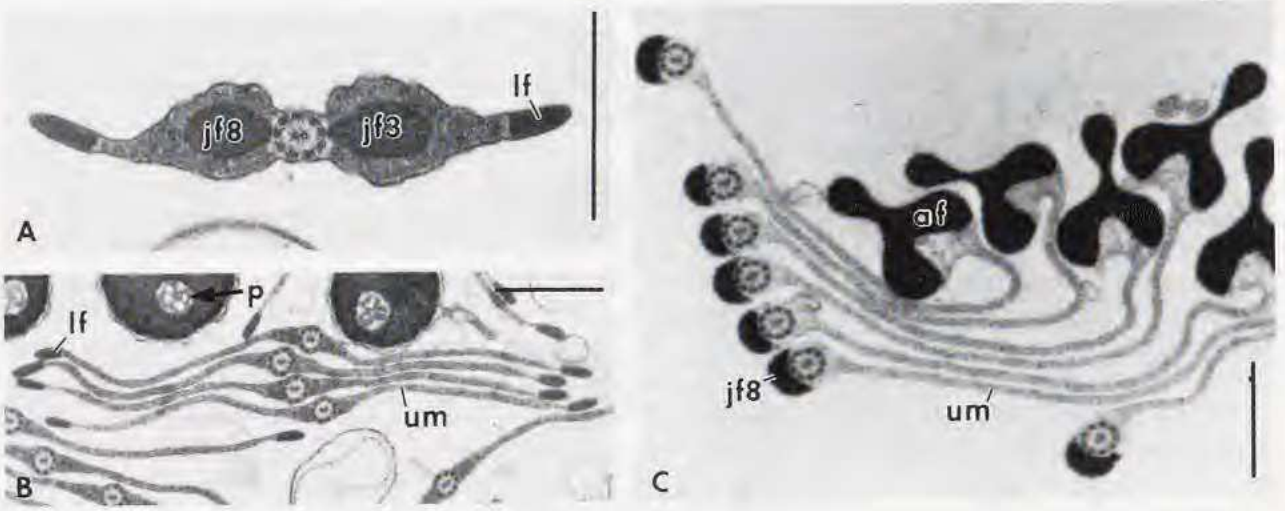


FIG. 4. — **A:** *Neoceratodus forsteri*. Transverse section through the anterior region of the tail of the spermatozoon. There is a large dense rod or fibre on each side of the axoneme, at doublets 3 and 8, and each rod is continuous with a 'fin' which terminates with a smaller, lateral density or fibre. **B:** Transverse sections of the axonemes more posteriorly where the fin is sufficiently extensive to be termed an undulating membrane. Three transverse sections of nucleus and endonuclear canals with contains perforatoria are also seen in the top of the figure. **C:** *Taricha granulosa*. Transverse sections through sperm tails showing urodele features of a longitudinal juxta-axonemal fibre at doublet 8, a long undulating membrane, and, in salamandroids, as in plethodontids, the Y-shaped transverse section of the axial (major) fibre. Abbreviations: af, axial (major) fibre; jf3 and jf8, juxta-axonemal fibres at doublets 3 and 8, respectively; lf, lateral fibre; p, perforatoria in the endonuclear canal of the nucleus; um, undulating membrane. Scale bar = 1 μm.

In *Neoceratodus* the anterior region of the sperm axoneme (not merely within the cytoplasmic canal as previously reported [29]) has a large dense rod on each side, at doublets 3 and 8, and each rod is continuous with a 'fin' which terminates with a further, smaller density (Fig. 4A, B). It is tempting to recognize homology between each fin and an amphibian sperm undulating membrane, between the large dense rods at doublets 3 and 8 and the amphibian juxta-

axonemal fibres and between the terminal densities (lateral fibres in Fig. 4A, B) and the axial fibre. This, if valid, would suggest that lissamphibians have retained, from an ancestor shared with dipnoans (and with other sarcopterygian fish?), only one of two former, bilateral, undulating membranes, and only one of a former pair of axial rods, but that the two juxta-axonemal fibres of urodeles are a persistence of the paired ancestral condition, the fibre at doublet 8 normally being lost in the Anura.

The condition in ray-finned fishes (Actinopterygii) of two lateral fins (also at doublets 3 and 8) [21] could conceivably have been precursory to lissamphibian undulating membrane and to dipnoan fins but homoplasy cannot be ruled out as lateral axonemal fins occur also in some echinoderms and protostomes.

Ground plan of the ancestral tetrapod spermatozoon

To the ground plan sperm of the basal tetrapod can be attributed all of the features which have been ascribed above to the ground plans of the lissamphibian and amniote spermatozoa with the exclusion of the synapomorphies identified for each of these groups. Synapomorphies of the tetrapod sperm relative to a presumed common ancestor shared with dipnoans, as exemplified by *Neoceratodus*, are deduced to have been few: nuclear 'shoulders' were developed as in basal amniotes and lissamphibians; an annulus (persisting in amniotes and possibly as the 'ring' in urodeles in the lissamphibians) may have developed or, alternatively, may have been retained from a pre-dipnoan ancestry. There were probably two longitudinal elements (dense fibres) peripheral to the axoneme adjacent to doublets 3 and 8, as in dipnoans, but these were apomorphically more extensive or, at least, well developed over a greater length, traversing much of the length of the axoneme. As noted above, a synapomorphy relative to *Neoceratodus* may have been loss of an undulating membrane between the fibre at 8 and the axoneme, leaving an albeit initially short undulating membrane between the fibre at 3 and the axoneme. The fibre at 8 would have persisted close to the axoneme. Plesiomorphic features deduced for the tetrapod sperm ground plan are the short distal centriole, and mitochondria, probably few immediately behind the nucleus, which had linear cristae as in lissamphibians. It appears unlikely that there were nine dense fibres as in amniotes.

Synapomorphies of higher amniote taxa

Chelonia and Sphenodontida. Turtles and *Sphenodon* conform to the hypothetical plesiomorphic amniote sperm and no convincing apomorphy seems demonstrable for the sperm of *Sphenodon* relative to the Chelonia.

Crocodilia. The ground plan for the Crocodilia, as exemplified by *Crocodylus johnstoni*, is very similar to that of the Chelonia and *Sphenodon*. All three have two or more endonuclear canals and concentric cristae with intramitochondrial bodies. Reduction to one perforatorial rod in *Caiman crocodylus* and *Crocodylus johnstoni* and restriction of the endonuclear canal to the anterior region of the nucleus in *Caiman* (though requiring confirmation) are apomorphies of the Crocodilia relative to Chelonia and *Sphenodon*. However, a possible synapomorphy of crocodiles, seen in *Caiman crocodylus* [45] and *Crocodylus johnstoni* (Fig. 1K) is investment of the two central singlets of the axoneme or of the distal centriole in a thick dense sheath which differs from the density, resembling a fibre, associated with the singlets in Chelonia, *Sphenodon* [16] and (homoplasically?) snakes [23, 35].

Aves. Loss of the subacrosomal cone may be a synapomorphy of birds as a whole. If they are the sister-group of crocodiles, they have apomorphically lost the concentric mitochondrial cristae. Ratites, though forming a monophyletic clade, have appeared paraphyletic [22] relative to non-ratite birds+monotremes but the three synapomorphies unifying the latter clade were insubstantial and probably artefactual. Monophyly of ratites cannot be considered proven,

however, as features considered to unify them (conical acrosome, fibrous sheath, and elongate centriole) [3] are all symplesiomorphies. Those in the earlier, cladistic analysis (supposed reversion to a long endonuclear canal, loss of the subacrosomal cone) [22] were also unconvincing and were associated with a computed paraphyletic origin of birds, in which ratites appeared as the sister group of galliforms+monotremes. If, however, the long endonuclear canal in more primitive ratites is a plesiomorphy carried over from basal amniotes, restriction of the canal to the anterior region of the nucleus in other non-passerines and passerines may be a synapomorphy of these, albeit homoplasic with crocodiles and derived ratites (emu). The avian feature of adhesion of all nine dense fibres to their axonemal doublets is also seen in monotremes; that it is apomorphic is debatable.

Squamata. Squamates are unified and distinguished by striking spermatozoal synapomorphies: loss of endonuclear canals and restriction of the perforatorial rod to a prenuclear location; development of intermitochondrial bodies (apparently from intramitochondrial bodies of lower amniotes); forward extension of the fibrous sheath into the midpiece; probably the paracrystalline structure of the subacrosomal cone; and, homplasically with other groups, shortening of the centriole from the elongate basal amniote condition.

Mammalia. Loss of the perforatorium (and endonuclear canal), homoplasic with some non-ratite birds, and great reduction of the distal centriole are synapomorphies jointly diagnosing mammal sperm. Separation, in marsupials and eutherians, of the peripheral dense fibres from their doublets with the exception that those at 3 and 8 may be attached to their doublets (the reverse of the situation in 'reptiles'), may be a therian synapomorphy.

ACKNOWLEDGEMENTS

I am very grateful to Linna DADDOW, David SCHELTINGA and Christopher TUDGE for excellent technical assistance. For access to material I thank Dr. Anne KEMP (*Neoceratodus*), Dr. Harry GRIER (*Taricha*), Dr. Arthur GEORGES (*Emydura*), Dr. Alison CREE (*Sphenodon*), Tony TUCKER (*Crocodylus*), Simon OLIVER (*Lampropholis*) and Professor John D. PETTIGREW (*Rhinolophus*).

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The Ultrastructure of Spermatozoa of the Squamata (Reptilia) with Phylogenetic Considerations

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ABSTRACT

Comparative ultrastructure of squamate families is reviewed, with new data for the South African chamaeleon, *Bradypodion karroicum*. Parsimony analysis is conducted, using Chelonia as the outgroup and branch and bound searching. Two major spermatozoal autapomorphies for the Squamata are extension of the fibrous sheath into the midpiece and (not computed) the paracrystalline subacrosomal cone. Further synapomorphies defining the Squamata *sensu strictu* are a single perforatorium in place of the two or three of Sphenodontida and Chelonia; loss of the endonuclear canal; presence of sinuous mitochondria (possibly an artefactual parsimony resolution as a columnar form is intuitively preferred); intermitochondrial location of dense bodies (mitochondrial transformations); presence of a well developed epinuclear electron lucent region and, equivocally, arrangement of the dense bodies as periodic rings. A major inference is polyphyly of the 'Sauria', the Scincomorpha and the Scincidae. Sphenomorphus group and egernid skinks show no close relationship to Eugongylus-group skinks which form the sister-group of the pygopodid *Lialis*. Snakes are the sister-group of the Eugongylus+pygopod clade. Gekkonidae appear to be a relatively plesiomorphic group, separated by several families from the Pygopodidae. The Iguania is not a monophyletic assemblage, iguanids and *Pogona* occur in the same clade but *Pogona* appears to be the sister-taxon of *Varanus*. Another iguanian, the chamaeleon *Bradypodion*, has an unresolved relationship with the gekkonid+snake+pygopodid+Eugongylus-clade. Sphenomorph and egernid skinks form an unresolved clade with *Chalcides* and lacertids but linkage of lacertids with the Teiidae in the Lacertoidea is not upheld. Pending further investigations of a larger number of taxa, these results can only be considered heuristic.

RÉSUMÉ

L'ultrastructure des spermatozoïdes des Squamata (Reptilia) et considérations phylogéniques

Ce chapitre comprend une synthèse de l'ultrastructure comparée des familles de Squamata, avec des observations nouvelles sur le Caméléon d'Afrique du Sud *Bradypodion karroicum*, et une analyse de parcimonie utilisant les Chelonia comme outgroup et une recherche par branch and bound. Les deux autapomorphies majeures des spermatozoïdes pour les Squamata sont l'extension de la gaine fibreuse dans la pièce intermédiaire et (n'intervenant pas dans le calcul) le cône subacrosomien paracristallin. D'autres synapomorphies définissant les Squamata *sensu strictu* sont un perforatorium unique à la place de deux ou trois chez les Sphenodontida et les Chelonia; la perte du canal endonucléaire; la présence de mitochondries sinueuses (ce qui pourrait donner lieu à un artefact de résolution car la forme en colonne est préférée de manière intuitive); la position intermitochondriale des corps denses (transformations mitochondriales); la présence d'une région épinucléaire claire aux électrons bien développée, et, de manière équivoque, la disposition des corps denses en anneaux périodiques. Une conséquence majeure est la polyphylie des "Sauria", des Scincomorpha et des Scincidae. Le groupe de Sphenomorphus et les Scincidae ergenides ne montrent pas de relations proches avec les Scincidae du groupe de Eugongylus, qui forment le groupe frère du pygopodide Lialis. Les Serpents sont le groupe-frère du clade

JAMIESON, B. G. M., 1995. — The ultrastructure of spermatozoa of the Squamata (Reptilia).with phylogenetic considerations. *In*: JAMIESON, B. G. M., AUSIO, J., & JUSTINE, J.-L. (eds), Advances in Spermatozoal Phylogeny and Taxonomy. *Mém. Mus. natn. Hist. nat.*, **166** : 359-383. Paris ISBN : 2-85653-225-X.

Eugongylus+pygopodes. Les Gekkonidae semblent être un groupe relativement plésiomorphe, séparé par plusieurs familles des Pygopodidae. Les Iguania ne sont pas un assemblage monophylétique, les iguanides et *Pogona* se trouvent dans le même clade mais *Pogona* semble être le groupe-frère de Varanus. Les relations d'un autre iguanien, le caméléon *Bradypodion*, ne sont pas résolues avec le clade gekkonides+serpents+pygopodides+Eugongylus. Les Sphénomorphes et les Scincidae egernides forment un clade non résolu avec Chalcides et les lacertides mais la liaison des lacertides avec les Teiidae dans les Lacertoidea n'est pas soutenue. En attendant d'autres études sur un plus grand nombre de taxons, ces résultats peuvent seulement être considérés comme heuristiques.

There is no comprehensive well-corroborated phylogeny available for the Squamata [28]. As the utility of spermatozoal ultrastructure as a source of characters for phylogenetic analysis is well established [26, 27, 31, 32], JAMIESON *et al.* [37] and OLIVER *et al.* [45] attempted to shed light on squamate classification and phylogeny by a comparative study of spermatozoal ultrastructure which is reviewed and extended here.

The ultrastructure of spermatozoa or spermiogenesis has been studied, though often cursorily, in the major groups of squamate reptiles. Families studied are: Scincidae [8, 14, 21, 29, 34, 35, 37, 44]; Lacertidae [7, 11, 21]; Teiidae [19, 41, 42]; Iguanidae [22, 48]; Anolidae [10]; Tropiduridae [12, 13]; Agamidae [2, 9, 15, 16, 17, 18, 45]; Chamaeleonidae [52 (spermiogenesis only), and this account]; Varanidae [45]; Gekkonidae [21, 37, 46]; and Pygopodidae [25, 37]. A brief account of the kinetic apparatus of the sperm of *Amphisbaena darwinii* (Amphisbaenidae) and *Liolaemus weigmanii* (Iguanidae) by SOTELO & CENÓZ [50] is chiefly of historic interest and will not be reviewed here. The spermatozoa of Serpentes have been more thoroughly investigated, in terms of taxa examined, than those of other squamates [1, 3, 5, 6, 20, 21, 24, 33, 45, 46, 49].

The present account reviews squamate sperm, provides the first description of mature chamaeleonid sperm, for the rare South African chamaeleon *Bradypodion karroicum*, and gives a preliminary parsimony analysis.

MATERIALS AND METHODS

Testes and ducts were dissected from a euthanased specimen of *Bradypodion karroicum* (Chamaeleonidae). Processing of the tissues was as in [34]. A cladistic analysis was performed using the PAUP program of SWOFFORD [51] (for details see parsimony analysis).

RESULTS AND DISCUSSION

Comparative sperm ultrastructure

Spermatozoal ultrastructure in the Squamata is summarized in Tables 1 and 2.

Scincidae. Descriptions of the male gametes of the Scincidae include a description of the mature spermatozoon of *Chalcides ocellatus tiligugu* [21]; an account of spermiogenesis, with some description of mature, epididymal sperm, in the same subspecies [8]; brief descriptions of spermiogenesis in *C. ocellatus* [14, 29]; an account of development of the midpiece in *Eumeces laticeps* [44]; and descriptions, with phylogenetic considerations, by JAMIESON & SCHELTINGA, of the sperm of the sphenomorph skink *Nangura spinosa* [34] and of *Tiliqua scincoides*, and *Ctenotus taeniolatus*, with brief reference to *Anomalopus verreauxii* [35]. JAMIESON *et al.* [37]

FIG. 1. — A generalized spermatozoon (diagrammatic), in longitudinal and corresponding transverse sections, of the Sphenomorphus group of the Scincidae (*Nangura spinosa*, *Ctenotus taeniolatus*, *C. robustus*, *Anomalopus verreauxii*) and Egernia-group (*Tiliqua scincoides scincoides*). Scales of various components are only approximate. Regional zonation of the acrosome vesicle is shown only for the transverse sections. After [34, 37].

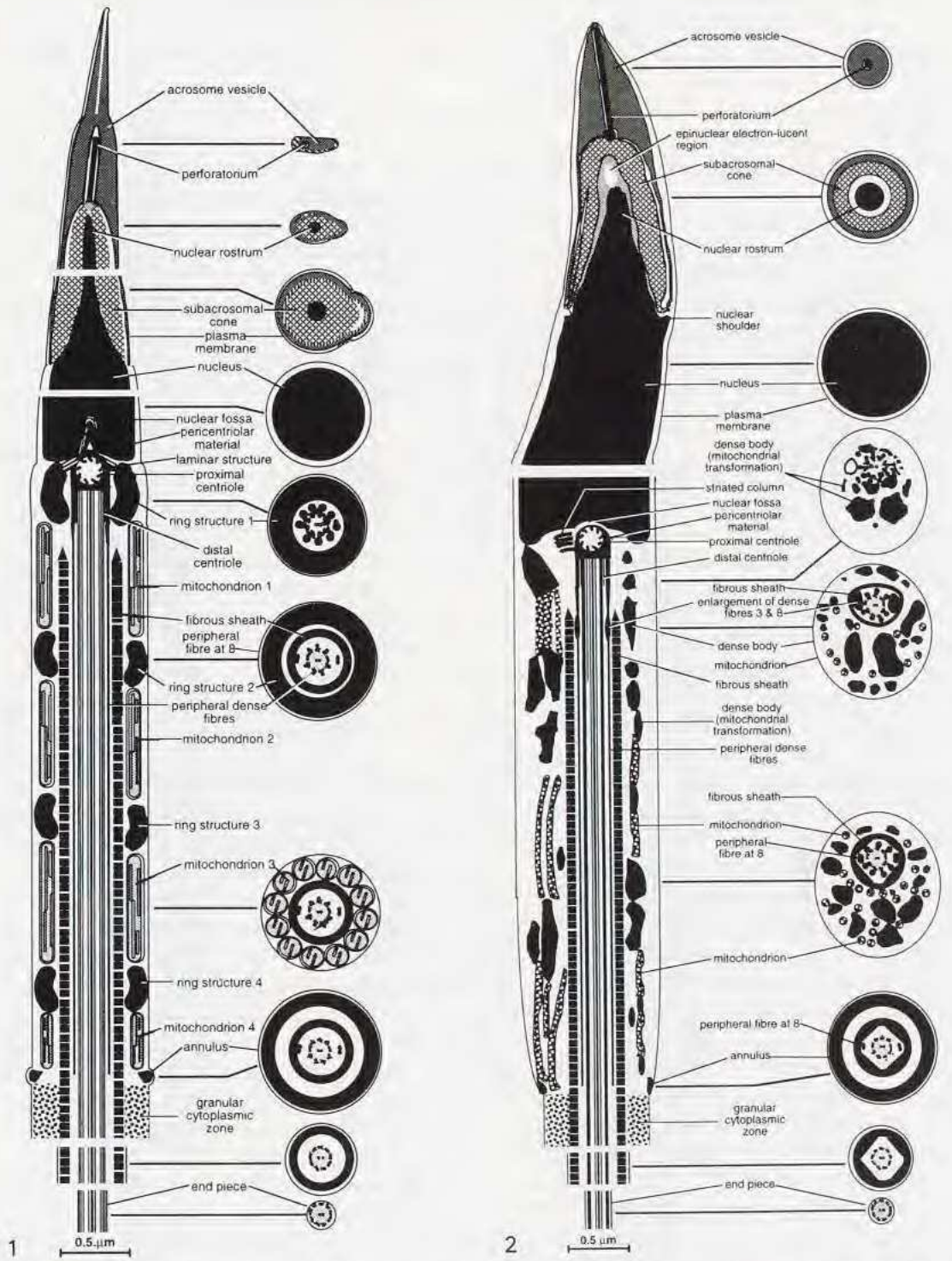

FIG. 2. — A generalized spermatozoon (diagrammatic) of the Eugongylus-group species of the Scincidae, in longitudinal and corresponding transverse sections. It is drawn from the sperm of *Carlia pectoralis* but is applicable also to *Cryptoblepharus virgatus* (Eugongylus-subgroup), and *Lampropholis delicata* (with *Carlia*, in the Lampropholis-subgroup). Scales of various components are only approximate. Regional zonation of the acrosome vesicle, though present, is not indicated. After [37].

have compared the sperm of *Ctenotus robustus,* a scincid of the Sphenomorphus group and other sphenomorphs (*Ctenotus taeniolatus*, *Nangura spinosa*), with those of *Cryptoblepharus virgatus*, *Lampropholis delicata*, and *Carlia pectoralis*, in the Eugongylus-group, in the classification of GREER [23].

Spermatozoa of skinks (e.g. *Ctenotus robustus*, *Carlia pectoralis*, *Cryptoblepharus virgatus*, and *Lampropholis delicata*) conform with other squamate sperm in the following respects: the sperm are filiform; the acrosome vesicle is in the form of a hollow, concentrically zoned cone which basally overlies a paracrystalline subacrosomal cone which invests the tapered anterior end of the nucleus; the perforatorium is a slender rod extending anteriorly from the subacrosomal material; the midpiece terminates with an annulus; peripheral dense fibres are associated with the 9 triplets of the distal centriole and the doublets of the axoneme within the midpiece; the peripheral fibres adjacent to doublets 3 and 8 are enlarged and each forms a double structure associated with the annulated fibrous sheath; usually all nine peripheral fibres are absent from the principal piece though in *Lampropholis delicata* they remain well developed in its anterior region and fibres 3 and 8 sometimes extend into the endpiece; the fibrous sheath extends anteriorly into the midpiece (squamate autapomorphy).

The sperm of species of the Sphenomorphus group (e.g. *Ctenotus*) and the Egernia-group (*Tiliqua*) (Fig. 1) differ from Eugongylus-group species (*Cryptoblepharus virgatus*, *Lampropholis delicata* and *Carlia pectoralis*) (Fig. 2), in the classification of GREER [23], in the following features: (1) the acrosome is elongate (it is relatively short in Eugongylus-group species); (2) the acrosome is depressed near its tip; (3) the perforatorium is strongly oblique (it is very slightly oblique in Eugongylus-group species); (4) a conspicuous laminated structure is present on each side of the proximal centriole (it is absent, though possibly represented by striated column(s) in Eugongylus-group species); (5) the midpiece is shorter absolutely and relative to the nucleus; (6) the midpiece has four dense ring structures in longitudinal succession (in Eugongylus-group species mitochondrial transformations are scattered irregular dense bodies of varying sizes); (7) mitochondria between the mitochondrial transformations form columnar structures in a circle around the fibrous sheath with numerous predominantly longitudinal cristae (in Eugongylus-group species mitochondria are elongate, tubular structures, with indistinct cristae, and weave between the intermitochondrial bodies); (8) enlargement of the peripheral fibres adjacent to doublets 3 and 8 occurs, as in all squamates, but not the gross enlargement which occurs in the anterior region of the axoneme in *Carlia* and *Lampropholis*.

From microcomplement fixation of albumin, BAVERSTOCK & DONNELLAN [4] showed the Eugongylus-group to be monophyletic as suggested by sperm ultrastructure [35].

The sperm of the European scincid species *Chalcides ocellatus tiligugu* [21] conforms closely to the description given above for the Sphenomorphus group, particularly in having a longitudinal series of four dense rings alternating with columnar mitochondria. An annulus was not described but absence is doubtful in view of its presence in all squamates which have been examined by the author. *Ch. ocellatus tiligugu* differs from the Sphenomorphus group in the composition of the dense rings which are shown diagrammatically as each being composed of large juxtaposed granules in single file. CARCUPINO *et al.* [8] describe the ring structures as "four rings of electron-dense material" and do not mention a granular composition.

For the scincid *Eumeces laticeps*, OKIA [44] described a midpiece with nine mitochondrial columns around the axoneme, a condition reminiscent of that in sphenomorphs. These "columns lie segmented by partial or complete rings of dense material" but it is not clear whether their arrangement conforms to the sphenomorph pattern.

A suite of character states which JAMIESON & SCHELTINGA [35] noted for the Sphenomorphus and Egernia-groups of the Scincidae is also seen in the teiid lizard, *Cnemidophorus sexlineatus* [41, 42] (see below): 1. Anterior depression of the acrosome. 2. The conical nuclear fossa containing dense material projecting from the proximal centriole. 3. The

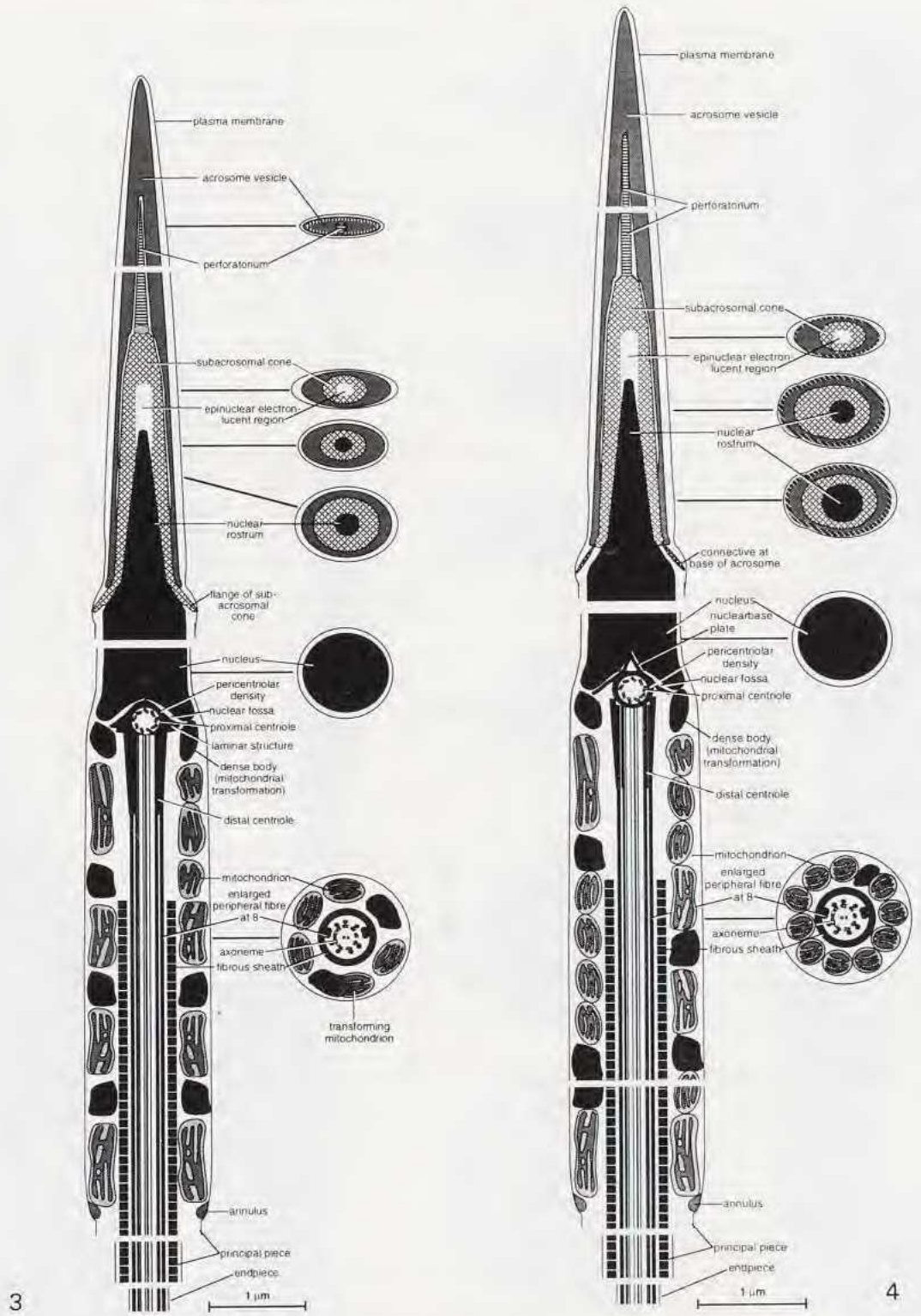

FIG. 3. — *Pogona barbata* (Agamidae). A diagrammatic representation of the spermatozoon, in longitudinal and corresponding transverse sections. After [37].

FIG. 4. — *Bradypodion karroicum* (Chamaeleonidae). A diagrammatic representation of the spermatozoon, in longitudinal and corresponding transverse sections. Original.

presence of a laminar structure extending from the pericentriolar apparatus (possibly unilateral in the teiid, and possibly homologous with the striated column(s) in Eugongylus-group sperm). 4. Presence of four, or in *Cnemidophorus* five, intermitochondrial rings alternating with columnar mitochondria. 5. The absence of sharply defined nuclear shoulders. 6. The apparent wide separation of the plasma membrane from the fibrous sheath in the anterior region of the principal piece. However, features 3 and 6 require confirmation for teiids.

The parsimony analyses (below) which are necessarily preliminary in view of our imperfect knowledge of these and other characters across the spectrum of squamate taxa, strongly suggest that skinks are not monophyletic and that sphenomorph-egernid and Eugongylus-group skinks are two distinct groups of squamates, the Eugongylus-group appearing to be the sister-group of pygopods (Fig. 10). As snakes form the sister-taxon of these two groups, it would appear that the legless condition has been independently derived in snakes as compared with pygopods, though as a parallelism from an ancestor shared also with Eugongylus-group skinks.

Lacertidae. Spermatozoa of the Lacertidae have been examined ultrastructurally in *Lacerta sicula campestris*, *L. lepida lepida*, *L. laevis*, *L. viridis* and *Algyroides alleni* [21], *L. vivipara* [11] and, with reference to development of the sperm head, *Podarcis* (=*Lacerta*) *taurica* [7]. They are morphologically very similar and resemble those of the sphenomorph-egernid Scincidae in many respects [21]. The head is curved and depressed [7, 11, 21]. The perforatorium ("apical groove") is oblique and the subacrosomal cone is paracrystalline [7, 21] as is typical of squamates. The midpiece is distinctive in having only two sets of dense bodies, the first has the appearance of two opaque masses on each side immediately posterior to the nucleus [21] and termed the "nuclear plate" [11]; this is probably correctly regarded by NEWTON and TRAUTH [42] as the equivalent of the first ring structure in teiid sperm. The second is a large ring (more certainly equivalent to ring structures of sphenomorph skinks) and questionably regarded as a "chromatoid body" [11], between the mitochondria in the distal third of the midpiece. In *L. vivipara*, there are only three tiers of mitochondria in longitudinal section and the intermitochondrial ring lies between mitochondrial tiers 2 and 3 [11]. The appearance and size of the dense bodies varies from species to species but not clearly enough to be used as a defining characteristic. The internal surface of the posterior ring is indented by numerous niches containing small masses of moderately opaque material [21]. In the order of 10 mitochondria are illustrated in transverse section for *L. lepida* [21] but only five in *L. vivipara* [11]. Though said to be filamentous [21], the mitochondria appear compact and only a few times longer than wide, with linear cristae. Peripheral dense fibres at 3 and 8 are enlarged and fused with or adpressed to the fibrous sheath [11, 21]. In mature sperm no annulus is indicated diagrammatically but in a micrograph a very small, weakly developed annulus appears to be present [21], and it is present in the late spermatid [11]. There are two centrioles and the implantation fossa is compact and rounded [11, 21].

Teiidae. The subacrosomal rod (putative perforatorium) has been shown in *Cnemidophorus lemniscatus lemniscatus* to develop from a subacrosomal granule [19]. The spermatozoon of

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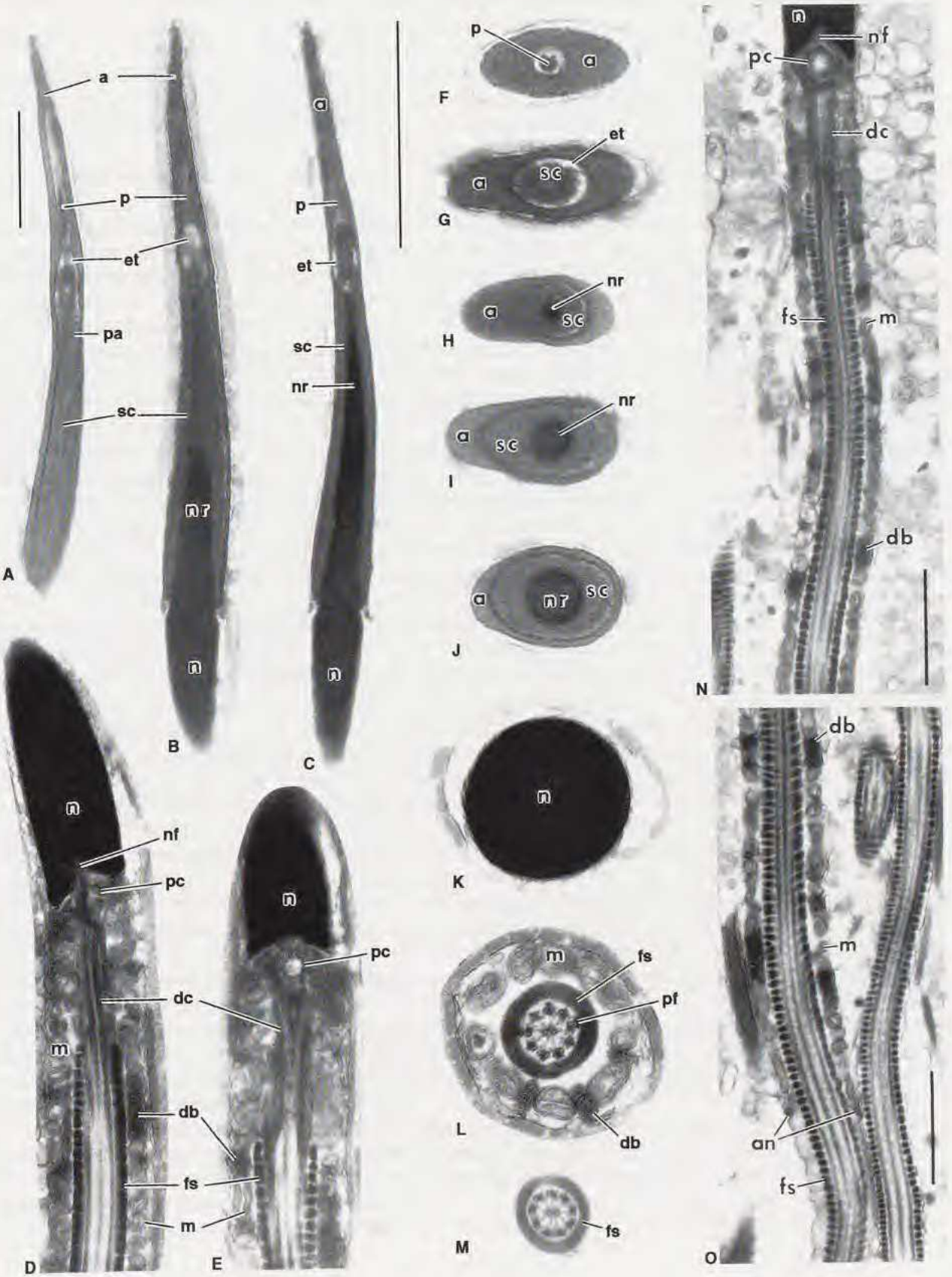
FIG. 5. — *Bradypodion karroicum* (Chamaeleonidae). **A, B, C:** Longitudinal sections (LS) of acrosome and anterior region of nucleus. **D, E:** LS of posterior region of nucleus and anterior region of midpiece. **F-M:** Successive transverse sections through F-J, the acrosome, K, the nucleus, L, the midpiece and M, the principal piece. **N:** LS centriolar region and midpiece. **O:** LS posterior region of midpiece, including annulus, and anterior region of principal piece. a = acrosome vesicle; an = annulus; db = dense body (mitochondrial transformation); dc = distal centriole; et = electron lucent space; fs = fibrous sheath; m = mitochondria; n = nucleus; nf = nuclear fossa; p = perforatorium; pc = proximal centriole; pf = peripheral dense fibre (coarse fibre); pm = plasma membrane; sc = subacrosomal cone. Original. Scale bar 1 µm.



C. sexlineatus has been the subject of detailed description [41, 42], augmented here by reference to published micrographs. A suite of characters shared with the Sphenomorphus and Egernia-group Scincidae is discussed under that family, above.

In *C. sexlineatus*, the acrosome caps and invests the anterior 1 μm of the nucleus which forms a pointed nuclear rostrum. The acrosome is depressed and spatulate, as in Sphenomorphus and Egernia-group scincids. Although an acrosome vesicle is recognized, no subacrosomal cone is described. Instead, a large vesicle and a more basal small vesicle are described posterior to the main part of the acrosome vesicle. From a micrograph there seems some possibility that the large and small vesicles represent subacrosomal material. It appears that a basal extension of the acrosome vesicle envelops this material, as in typical squamate and, indeed, tetrapod spermatozoa. This interpretation is supported by presence of an electron dense connective between the posterior acrosome and the cell membrane. A well developed rodlike perforatorium, composed of longitudinal fibres, possibly in helical array, is present. It does not appear to be oblique but is unusual in being strongly eccentric. A narrow epinuclear electron lucent zone is visible anterior to the rostrum. The nucleus is curved and strongly condensed and has a low, conical, basal implantation fossa. The absence of sharply defined nuclear shoulders at the base of the rostrum is a resemblance to the above-mentioned scincids. The midpiece consists of five tiers, usually, of shortly columnar mitochondria separated by intermitochondrial dense "ring structures" and terminating with an annulus, giving the formula rs1/mi1, rs2/mi2, rs3/mi3, rs4/mi4, rs5/mi5, an. Each mitochondrial set consists of 8 to 10 (usually 9) mitochondria arranged around the axoneme. The axoneme is surrounded by the fibrous sheath from rs3 to the posterior end of the principal piece, leaving a 0.7 μm endpiece. The A subtubules of the 9+2 axoneme are filled with dense material. A transverse section of the distal centriole (basal body) shows coarse fibres enveloping the triplets, extending both peripherally and internally to each triplet, and, as usual, displaced clockwise into the inter-triplet space. Dense material is associated with the two central singlets. Coarse fibres are absent from illustrated transverse sections of the principal piece. Longitudinal extracellular tubules surround the nucleus and flagellum in the ductus deferens. The suggested presence of a laminar structure extending from the pericentriolar apparatus, equivalent to the bilateral structure in scincids [35], requires confirmation.

Iguanidae. The account by FURIERI [22], based chiefly on three species of the Iguanidae, *Cupriguanus scapulatus*, *Phymaturus palluma* and *Liolaemus austromendocinus*, is summarized here, using the terminology employed in the present account. The general structure of the spermatozoon is much as described for skinks, with the following details. The acrosome is circular in cross section; the single, well developed perforatorium appears to have a pointed, not square, tip and to lack a basal plate but this requires confirmation. An endonuclear canal is absent. The subacrosomal cone ("inner cap") is paracrystalline and surrounds a pointed nuclear rostrum which is preceded by an epinuclear electron lucent region. The nucleus is elongate, with condensed chromatin, and has a shallow basal fossa which houses the anterior portion of the proximal centriole; this is at an angle to the distal centriole. There are distinct nuclear shoulders, apparently intermediate in shape between the sharp and rounded forms. The midpiece is relatively short, and, with a length of 7 μm, is much shorter than the head. Although the mitochondria, which are long, thin and numerous, are arranged in a regular circlet around the axoneme and fibrous sheath, they form helices, with a regular arrangement in *C. scapulatus* and especially *L. austromendocinus*. They have a very fine calibre in *L. austromendocinus*, thus being reminiscent of those of snake sperm. The quantity and arrangement of the dense intermitochondrial bodies are characteristic of each of the three species. In *Phymaturus*, the mitochondrial sleeve is subdivided into six long sections, separated by contiguous small masses of opaque material arranged in a ring which is sometimes not completely closed. A sixth dark ring is present at the beginning of the mitochondrial sleeve and a seventh, much smaller ring (here considered to be the annulus) closes the sleeve, giving the formula, in the system of NEWTON and

TRAUTH [42], of rs1/mi1, rs2/mi2, rs3/mi3, rs4/mi4, rs5/mi5, rs6/mi6, an. In *Cupriguanus*, the dense material is more scanty and does not seem to form closed rings. In *Liolaemus*, the dense bodies form small dark plates which are few in number and are distributed without order. Development of nine peripheral axonemal fibres is limited to the short region of the midpiece anterior to the fibrous sheath. Within the fibrous sheath there are only two, enlarged fibres and these are joined to the sheath. These are said to lie at doublets 3 and 7 but the micrographs and diagram indicate that they are in the usual position adjacent to doublets 3 and 8.

Anolidae. The account of spermiogenesis in the so-called American chamaeleon, *Anolis carolinensis* (Anolidae) [10] reveals no characters which can conclusively be ascribed to the mature spermatozoon. However, the spermatid has the usual acrosome vesicle and subacrosomal material investing the nuclear rostrum; a condensed, curved nucleus; a low, dome shaped but apically pointed implantation fossa; and a proximal centriole tilted relative to the distal centriole. The fibrous sheath is posterior to the annulus but whether it later extends into the midpiece is not known, though likely.

Tropiduridae. Accounts of spermiogenesis in *Tropidurus torquatus* [12, 13] yield little information with regard to the mature spermatozoon. The following account is derived from text and micrographs. As usual for squamates, the acrosome vesicle and underlying subacrosomal cone ensheath a tapered anterior extension of the nucleus (nuclear rostrum). Smooth nuclear shoulders support the posterior end of the acrosome. There is a large lacuna within the nuclear rostrum, near it tip. The sperm head does not appear to be depressed. The very short midpiece has only three gyres of mitochondria. The statement that the fibrous sheath is found only in the principal piece, except for a few dense spots around the annulus (unlike any other known squamate sperm) is to be doubted as the observation is not derived from a mature spermatozoon. Peripheral dense fibres are limited to a short segment near the short neck cylinder which lies between the proximal centriole and the base of the nucleus. Multiple cytoplasmic membranes around the acrosome tend to disappear by maturity whereas those in snakes and pygopods appear to persist. No evidence of dense bodies or ring structures is provided [13].

Agamidae. In the Agamidae, differentiation of the sperm head has been described for *Agama stellio* [2], *Uromastyx philbyi* [15], *Stenodactylus selvini* [16], for *A. adramitana* [18] and, for the nuclear manchette, *A. agama* [9]. Differentiation of the sperm tail has been described in *Uromastyx philbyi* [17]. The entire sperm has been described for *Pogona barbata* [45] (Fig. 3).

The mature spermatozoon of *Agama stellio* illustrated by AL-HAJJ *et al.* [2] conforms closely in ultrastructure to that of the cofamilial *Pogona barbata* [45]. Flattening of the acrosome is again seen [2], as in *A. adramitana* [18], but supposed flattening of the nucleus is not supported by micrographs. A well defined narrow acrosomal cortex is present (see also [8, 18]). From published accounts [37], division of the acrosome into cortex and medulla can be claimed not only for the Agamidae but also for Lacertidae, Teiidae, Chamaeleonidae, Varanidae and all families of the Serpentes. This subdivision is not apparent in Gekkonidae but it is possible that layering described for sphenomorph skinks [34] and the anterior saccular enclave seen in the Eugongylus-group skink *Lampropholis delicata* and in the pygopod *Lialis burtonis* [37] is equivalent. The wide zone between the acrosomal cortex and the perforatorium [2, 18] is clearly the subacrosomal cone. Late spermiogenic stages of *A. agama* [9] confirm the circular nuclear section but flattened acrosome and wide, distinct nuclear shoulders which, as in *Pogona barbata*, are intermediate between the concave, angular shoulders of, for instance, Eugongylus-group scincids, and the rounded shoulders of sphenomorphs.

With regard to affinities of the sperm of *Pogona barbata*, it shares a larger number of character states with the sperm of *Varanus gouldii* than with other non-agamid squamate taxa which have been studied. Those also common to the sphenomorph-teiid-varanid assemblage have been indicated above.

A varanid-agamid relationship does not appear to have been suggested on the basis of somatic morphology (see review by RIEPPEL [47]). Of the three noteworthy spermatozoal similarities of depressed acrosome, alternating, intermitochondrial rings and the not entirely similar basal plates, only the knob-like form of the basal plate appears to be an agamid-varanid synapomorphy and it is homoplasic with Eugongylus-groups skinks. Nevertheless, the validity of recognizing a varanid-agamid relationship deserves further consideration.

A number of features shared between *Pogona barbata* and sphenomorph skinks, *Tiliqua scincoides* (Egernia-group), *Cnemidophorus sexlineatus* (Teiidae) and *Varanus* appear, on intuitive consideration and in parsimony analysis, to be symplesiomorphic for these taxa: the single, pointed perforatorium and linear cristae (plesiomorphic for squamates); the epinuclear electron lucent zone, the elongate, cylindrical nucleus (plesiomorphic for tetrapods); absence of multilaminar membranes around the midpiece (presence has recently been recognized as a similarity and possible synapomorphy of snakes and pygopods [37, and this account]; and extension of the fibrous sheath into the midpiece (autapomorphy of squamates).

Chamaeleonidae. A preliminary study of the spermatozoon of the rare South African chamaeleon, *Bradypodion karroicum* (Figs 4, 5), reveals close similarity, on intuitive consideration, to the spermatozoa of the Agamidae (see above). The acrosome is sharply attenuated but is depressed in one plane, The acrosome vesicle is divisible into cortex and medulla. Within the acrosome medulla the subacrosomal space encloses a perforatorium in the form of a narrow cylinder which tapers to a blunt point anteriorly and is cross striated. A basal plate, questionably present in *Pogona barbata*, is absent. The subacrosomal cone, occupying the basal two thirds of the length of the acrosome, is conspicuously paracrystalline. The cylindrical, electron dense nucleus has slight, smoothly rounded shoulders and a long, slightly curved nuclear point (rostrum) which projects almost to the tip of the subacrosomal cone and is preceded by an electron lucent epinuclear region. There is a broad basal nuclear fossa which, in being pointed anteriorly, resembles that of sphenomorph skinks more closely than the shallow rounded fossa of *Pogona*. The proximal centriole, consisting of nine triplets, lies at right angles to the long axis of the sperm with its anterior region within the nuclear fossa (implantation fossa); a narrow density anterolateral to this centriole appears to continue to the summit of the fossa. As in agamids, a distinct lamellar structure is not discernible. As in *Pogona*, the distal centriole, forming the basal body of the axoneme, is elongate but similarly does not extend into the fibrous sheath. In transverse section of the midpiece through the fibrous sheath approximately 10 circular mitochondrial profiles form a single circlet around the sheath. In longitudinal sections, the profiles are again mostly circular but may show elongation consistent with a spiral arrangement and this is particularly true of the mitochondria in the short region of the midpiece anterior to the fibrous sheath. Small intermitochondrial dense bodies sporadically interrupt the mitochondria in transverse and longitudinal sections. Dense rings of the type seen in sphenomorph skinks are not present. A large dense granule is visible in some mitochondria and there is evidence of transformation of mitochondria into intermitochondrial dense bodies. The midpiece is moderately elongate and terminates at a distinct if small annulus. In the midpiece the axoneme has ninr coarse fibres; those at 3 and 8 are enlarged, appear double, and are adpressed to the fibrous sheath. No coarse fibres are visible in the principal piece, posterior to the mitochondrial midpiece. For a considerable distance posterior to the annulus a wide band of cytoplasm surrounds the fibrous sheath as in *Pogona barbata* and *Varanus gouldii* [45] but also in Eugongylus, sphenomorph, and egernid group skinks [35] and in the gekkonid *Heteronotia binoei* [37].

In the parsimony analysis (Fig. 10) chamaeleonids have an unresolved position, with gekkonids, at the base of the Eugongylus-group-pygopodid-snake clade. Their usual iguanian relationship or placement in the Agamidae is not upheld.

Varanidae. In view of the fact that only one varanid has been examined for spermatozoal

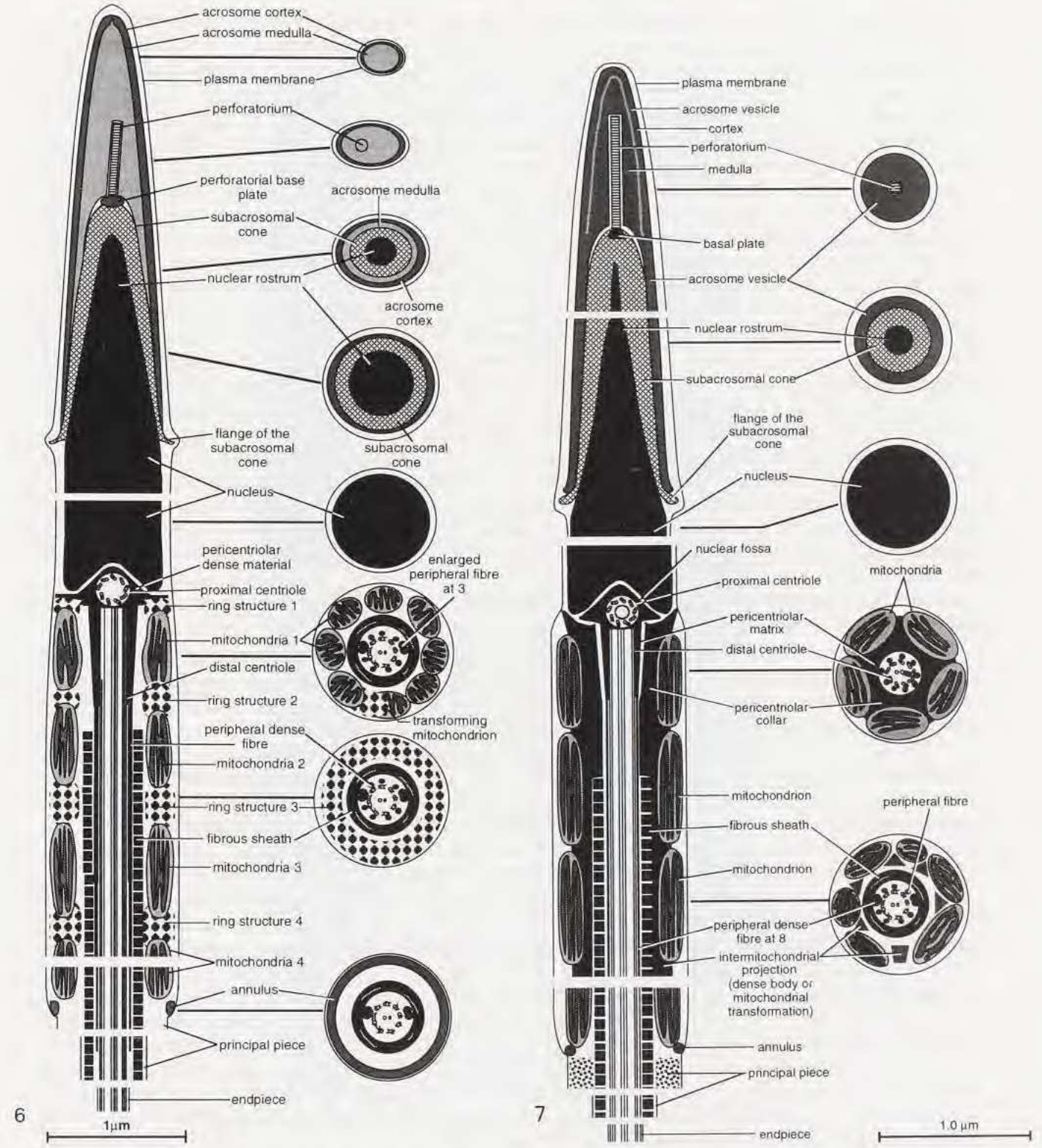


FIG. 6. — *Varanus gouldii flavirufus* (Varanidae). A diagrammatic representation of the spermatozoon, in longitudinal and corresponding transverse sections. After [45].

FIG. 7. — *Heteronotia binoei*. (Gekkonidae). A diagrammatic representation of the spermatozoon, in longitudinal and corresponding transverse sections. After [37].

ultrastructure (Fig. 6), the following comparison with the sperm of other squamates can be considered only a most preliminary indication of affinities of the family. A suite of character states has been described for the sperm of *Varanus gouldii flavirufus* [45] which is known elsewhere in squamates in the Sphenomorphus and Egernia (*Tiliqua*) groups of the Scincidae [37] and in the teiid lizard, *Cnemidophorus sexlineatus* [42] although some are also seen in agamids (e.g. *Pogona barbata* [45]). This suite includes the following states. 1. Anterior depression of the acrosome (also in agamids). 2. The conical nuclear fossa containing dense material projecting from the proximal centriole. 3. The presence of a laminar structure extending from the pericentriolar apparatus (possibly unilateral only in the teiid) although this is less definitely laminated in *V. gouldii*. 4. Presence of four intermitochondrial rings alternating with columnar mitochondria. The rings differ in *V. gouldii* in being composed of many large granules in contrast with the solid condensed structure in the sphenomorph-Egernia-group and in *Cnemidophorus*. The difference is lessened, however, by supposed constitution of the rings in the scincid *Chalcides ocellatus* from a single circlet of large granules [21]. (An alternation of mitochondria and albeit incomplete rings occurs in also in *P. barbata*). 5. The rounded rather than angular nuclear shoulders are apomorphic relative to the "sharp" or angular shoulders of *Sphenodon* [32] and primitive frogs [36]; a feature occurring only in the Eugongylus-group skinks [37]. (*P. barbata* shows an intermediate condition.) 6. The wide separation of the plasma membrane from the fibrous sheath in the anterior region of the principal piece (also in *P. barbata*), although this has not been demonstrated with certainty for *Cnemidophorus*.

In the parsimony analysis (Fig. 10) the alternation of ring structures with mitochondrial columns computes as an ambiguous basic synapomorphy of the squamates as a whole, rather than merely of the iguanid through sphenomorph assemblage. Flattening of the acrosome computes as the sole basal synapomorphy of this assemblage, independently developed in *Bradypodion*. Consideration may be given, however, to the possibility that flattening is basic to squamates and has been lost in the snake+pygopodid+Eugongylus-group clade.

Gekkonidae. In the Gekkonidae, FURIERI [21] briefly described the sperm of *Lygodactylus picturatus* and made reference to *Hemidactylus frenatus*, *H. mabouia*, and *Tarentola mauritanica mauritanica*; PHILLIPS & ASA [46] described formation of the midpiece in *Sphaerodactylus cinereus* and JAMIESON *et al.* [37] described the sperm of *Heteronotia binoei*.

In the spermatozoa of *H. binoei* (Fig. 7) no epinuclear electron-lucent region has been observed (computing as a loss); nuclear shoulders are smooth, as in sphenomorph skinks; mitochondria are large and discrete, arranged in a circle around the fibrous sheath, with intervening mitochondrial transformations, and extend longitudinally as slender columns. A feature not known in skinks is indentation of the median surfaces of the mitochondria at intervals by triangular dense bodies which are perhaps always longitudinally interconnected.

The sperm of *Lygodactylus picturatus*, *Tarentola mauritanica* and *Hemidactylus frenatus* [21] are generally similar to those of *H. binoei*, but the amount of intermitochondrial material (dense bodies or putative mitochondrial transformations of the present work) is greater in *Lygodactylus picturatus* and is present in decreasing amounts in *Tarentola mauritanica* and *Hemidactylus frenatus* respectively. The stellate arrangement of dense bodies, seen in cross section of the midpiece, in *T. mauritanica* closely resembles that in *H. binoei*. A stopper like electron dense perforatorial plate, seen in *H. binoei*, is illustrated for *T. mauritanica* and, less clearly, *L. picturatus*. A micrograph of the midpiece of *Sphaerodactylus cinereus* [46] shows a longitudinal series of four columnar mitochondria on each side (said to total 20 mitochondria for the midpiece) alternating with dense bodies, of comparable length, and the small annulus. In the absence of transverse sections it is difficult to compare this arrangement with that of other gekkonids but the columnar form of the mitochondria is reminiscent of that in *Heteronotia*.

Pygopodidae. The spermatozoa of *Lialis burtonis* (Fig. 8) are again like those of scincids in

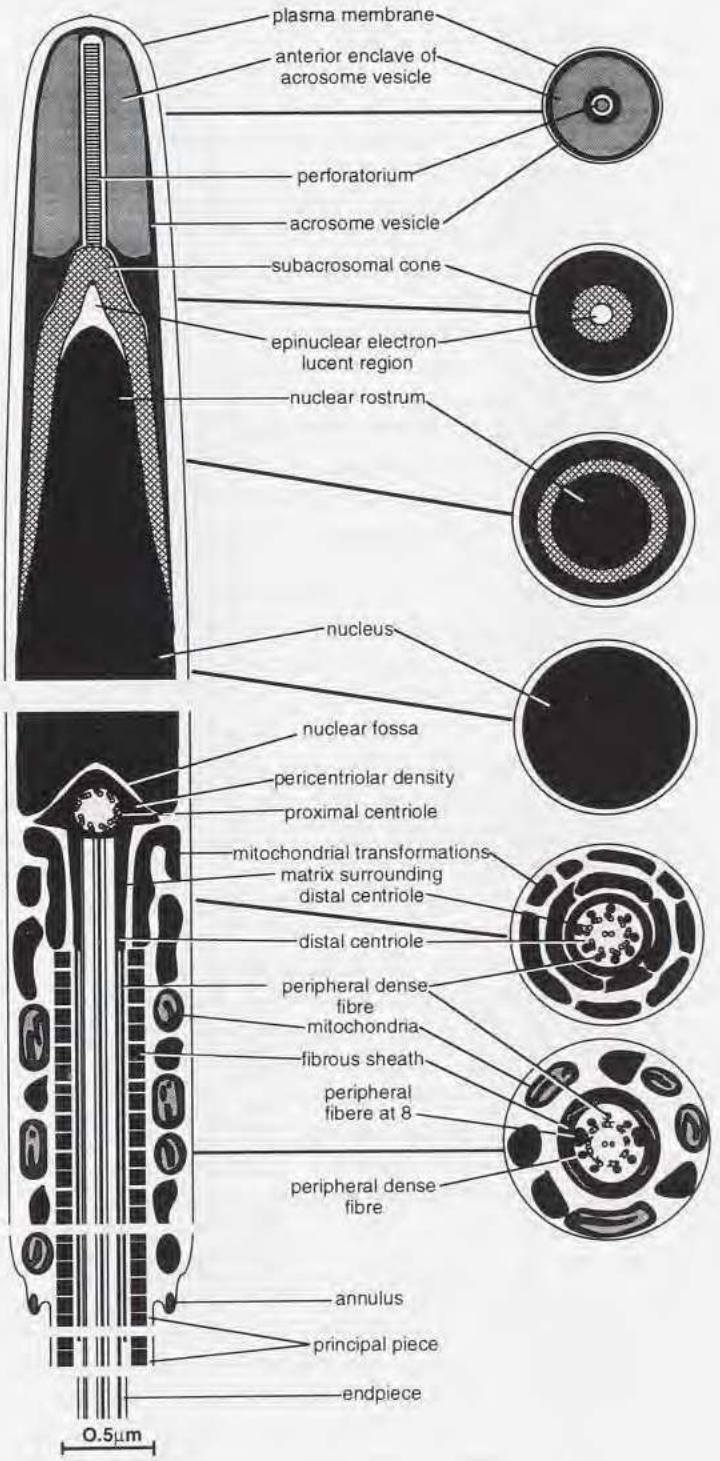


FIG. 8. — *Lialis burtonis* (Pygopodidae). A diagrammatic representation of the spermatozoon, in longitudinal and corresponding transverse sections. After [37].

their chief features, as was noted by HARDING *et al.* [25] in their preliminary report for the pygopods *Aprasia repens*, *Delma tincta*, *L. burtonis* and *Pygopus lepidopus*. However, in *L. burtonis* the acrosome is fore-shortened and apically domed and the perforatorium extends virtually to its tip; nuclear shoulders are absent; the mitochondria are small subspheroidal structures, four or five in a transverse section, and appear very numerous in longitudinal single file, alternating singly or in groups with one or more dense bodies, with evidence that they are sinuous; dense bodies also form an interrupted collar around the distal centriole [37]. The *L. burtonis* sperm shares a suite of apparently apomorphic character states of varying distinctiveness with Eugongylus-group skinks (*Cryptoblepharus virgatus*, *Carlia pectoralis* and *Lampropholis delicata*). They are as follows [37]. The perforatorium is square-ended rather than pointed. The midpiece is elongate, that in the Eugongylus-group species being less elongate but strikingly longer than that of sphenomorphs or other investigated squamates with the exception of the very long mitochondrial sheath of snake sperm [33, 45]. However, the mitochondria in *L. burtonis*, though forming a single layer as in snake sperm, are not as narrow. They are not irregularly interspersed with the dense bodies as they are in the Eugongylus-group skinks and are not as elongate as the mitochondria of the Eugongylus-group and snakes. Nevertheless, an elongated zigzag configuration much as in snake sperm is illustrated in a superficial longitudinal section of the midpiece of *Aprasia repens* by HARDING *et al.* [25] and these authors have noted a further striking similarity to snakes in the existence of a multilaminar membrane around the midpiece in their material of *L. burtonis*, and around the flagellum in *Delma tincta*. These multilaminar membranes had previously been considered unique to snake sperm [21, 33]. The co-occurrence of elongate, tubular, zigzagged mitochondria and an, albeit transient, multilaminar cell membrane in the sperm of pygopodids and snakes is here considered to warrant serious consideration that these two groups of legless squamates share a common origin despite lack of a direct sister-group relationship in the cladistic analysis (Figs 10, 11).

Serpentes. AUSTIN [3] gave a detailed account of the fine structure of the sperm tail in *Lampropeltis getulus*, *Coluber constrictor*, *Drymarchon corais*, *Crotalus adamanteus*, *Micrurus fulvius* and *Constrictor* sp., BOISSON & MATTEI [5, 6] described spermiogenesis in *Python sebae*. HAMILTON & FAWCETT [24] gave details of the neck and midpiece in *Lampropeltis getulus* and *Constrictor constrictor*, SAITA *et al.* [49] described spermiogenesis in *Coluber viridiflavius*, PHILLIPS & ASA [46] described the formation of the midpiece with reference to the behaviour of the annulus in *Masticophis flagellum flagellum* and AFZELIUS [1] described occlusion of microtubules in *Liophis miliaris*. Nevertheless, only FURIERI [20, 21] had given an account of the ultrastructure of the entire spermatozoon (giving in the latter work a general account for four species of Colubridae, *Coluber viridiflavius viridiflavius*, *Natrix tesselata tesselata*, *N. natrix*, and *Coronella austriaca*, and one species of Viperidae, *Vipera aspis aspis*) until the spermatozoon of *Nerodia sipedon* was described by JAMIESON & KOEHLER [33]. OLIVER *et al.* [45] describe the sperm of *Boiga irregularis*, *Stegonotus cucullatus* (Colubridae), *O. microlepidotus* (Elapidae), and *Aspidites melanocephalus* (Boidae).

Snake sperm (Fig. 9) present features [3, 20, 21, 24, 33, 45, 46] common to squamate sperm: they are filiform; the acrosome vesicle is in the form of a hollow, concentrically zoned cone which basally overlies a subacrosomal cone which invests the tapered anterior end of the nucleus; the perforatorium is a slender rod extending anteriorly from the subacrosomal material; the midpiece contains dense bodies (mitochondrial transformations) in addition to the mitochondria; the fibrous sheath surrounding the axoneme extends anteriorly into the midpiece (squamate autapomorphy); nine peripheral dense fibres accompany the triplets of the distal centriole and the doublets of the axoneme in the midpiece; the fibres adjacent to doublets 3 and 8 are enlarged, each as a double structure associated with the fibrous sheath; the endpiece lacks peripheral fibres and the fibrous sheath. A poorly developed "stopper-like" perforatorial base plate

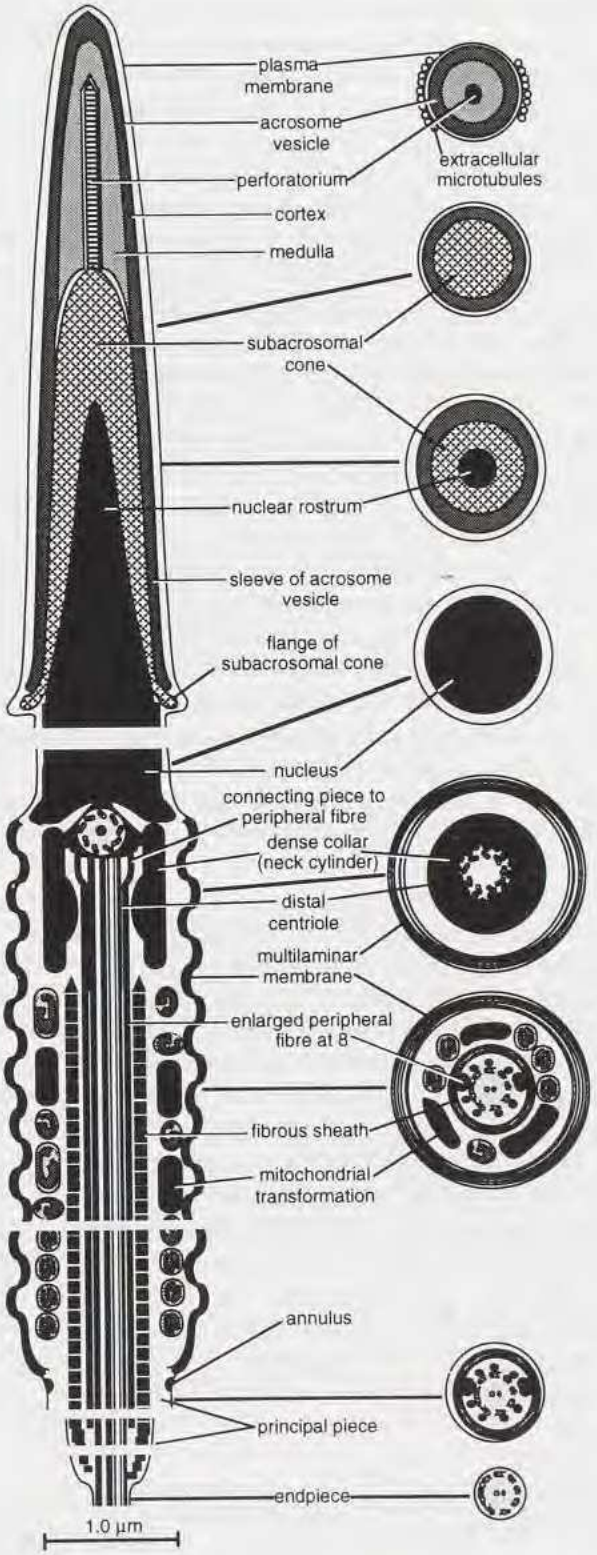


FIG. 9. — A diagrammatic representation of the spermatozoon of the Serpentes, in longitudinal and corresponding transverse sections. After [45].

in the colubrid *Nerodia sipedon*, unknown in other snakes, is presumably homoplasic with that of gekkonids. An electron-lucent space caps the nuclear point in the snakes *Boiga irregularis* and *Stegonotus cucullatus* as in some other squamate orders [33, 45] but is poorly developed.

Less widespread is a suite of apparently apomorphic characters states of snake sperm [33, 45] shared with Eugongylus-group skinks (*Cryptoblepharus virgatus*, *Carlia pectoralis* and *Lampropholis delicata*) and pygopodids. The shared states are as follows. The midpiece is greatly elongated in snakes (mitochondrial sheath of FURIERI [21]), that in the Eugongylus-group species, and pygopodids but also in gekkonids, being less elongate but strikingly longer than that of sphenomorph skinks or other investigated squamates. The mitochondria in at least some pygopodids [25] form zigzagged tubes as in snakes. They are not irregularly interspersed with the dense bodies as they are in the Eugongylus-group skinks. A multilaminar membrane is seen in some pygopods as in snakes (see above). JAMIESON *et al.* [37] stated that these similarities of snake, pygopodid and Eugongylus-group sperm warranted further investigation with a view to determining the degree of homoplasy as against synapomorphy. Individually these characters appear in some other squamates. Thus we have seen that extracellular tubules occur in immature teiid sperm and that multilaminar membranes invest the acrosome in immature tropidurid sperm. Possibly both types of structure are a normal feature of developing squamate sperm. Two apomorphies of snakes and pygopodids, the multilaminar membrane and the tubular zigzagged mitochondria are striking, and intuitively were considered synapomorphic resemblances. However, multilaminar membranes computed as basal not only to these two groups but also to Eugongylus-group skinks, in which they were computed as lost, and sinuous mitochondria parsimoniously (but not, perhaps, plausibly) computed as a basic squamate feature.

The evidence, albeit uncertain, for transformation of extracellular tubules into multilaminar membranes in *Boiga irregularis* possibly represents a stage in production of the membranes. Conversion of microtubules into membrane-like laminate appendages of testicular sperm is known in the Lepidoptera (see review in Jamieson [30]). It has been suggested that the multiple layers of membranes provide a source of endogenous phospholipid that could be utilized as a source of energy for motility [24].

The dense collar (termed the neck cylinder by AUSTIN [3]) is considered to be homologous with the intermitochondrial dense bodies [24, 45] which in turn have been shown in skinks (e.g. *Cryptoblepharus virgatus*) to be derived from mitochondria [37]. The dense element in the central axis of the proximal centriole, seen in *Aspidites*, is also illustrated and described for *Lampropeltis getulus* [24] and is stated to be regularly found in mammalian sperm. They [24] also observe the presence, unknown in other vertebrates, of extracellular microtubules, observed here in at least *Aspidites melanocephalus* and *Boiga irregularis*. With regard to the axoneme, they remind us that enlargement and prolongation of the peripheral fibres 3 and 8 in snakes (as, we note, in all squamates) is the reverse of the situation in the mammalian sperm tail where these are the smallest fibres and terminate first. The density associated with one of the two central singlets [3, 24], thought to be unique to snake sperm, has since been demonstrated for the skinks *Nangura spinosa* [34], *Tiliqua scincoides* and less certainly *Carlia pectoralis* [35], the gekko *Heteronotia binoei* [37] and the teiid *Cnemidophorus sexlineatus* [42] and may well be more widely demonstrated in squamates when favourable sections of the centriole are obtained. Dense material filling the A subtubules in the posterior portion of the axoneme has been demonstrated for doublets 1, 2, 5, 6 or 1, 2, 5, 6 and 7 in *Liophis miliaris* [1].

Parsimony analysis

A posteriori to intuitive consideration of comparative spermatozoal ultrastructure, with some inferred relationships, which has been detailed above, preliminary parsimony analyses have been

TABLE 1. — Ultrastructural characters of squamate sperm used in parsimony analysis. Character states numbered in parentheses are not transformation series as polarity was determined by use of an outgroup. The first state is nevertheless considered plesiomorphic.

Character	States
1 Acrosome TS	(0) circular (1) depressed,
2 Perforatorial base plate	(0) absent or indistinct (1) knoblike (2) stopperlike,
3 Perforatorial tip	(0) pointed (1) square ended,
4 Perforatoria, number	(0) two or more (1) one,
5 Epinuclear lucent zone	(0) absent (1) poorly developed (2) well developed,
6 Midpiece	(0) short (1) moderately long (2) very long,
7 Mitochondria in TS	(0) regular circlet (1) not regular (2) intermediate,
8 Mitochondria, shape	(0) rounded (1) columnar (2) sinuous tubes (3) 'intermediate rounded-columnar',
9 Dense bodies	(0) intramitochondrial (1) regular rings (2) scattered (3) linear series (4) stellate spiral (5) 2 groups,
10 Dense bodies, if regular	(0) not applicable (1) solid (2) granular (3) single file granules,
11 Cristae	(0) concentric (1) linear,
12 Nuclear shoulders	(0) sharp (1) rounded (2) absent,
13 Nuclear shape	(0) elongate (1) stout,
14 Endonuclear canal	(0) present (1) absent,
15 Fibres 3 and 8	(0) enlarged (1) grossly enlarged anteriorly,
16 Multilaminar membranes	(0) absent (1) present,
17 Fibrous sheath	(0) not in midpiece (1) in midpiece

performed using the PAUP program. The characters employed are those listed in Table 1 and the states of these characters are given in Table 2 which provides a summary of ultrastructure discussed in this account.

A branch and bound search was performed for the total data matrix (Table 2). The following options were applied: addition sequence: furthest; 1 tree held at each step during stepwise addition; MULPARS option in effect; steepest descent option not in effect; initial upper bound: unknown (compute via stepwise); branches having maximum length zero collapsed to yield polytomies; no topological constraints; trees unrooted; multi-state taxa interpreted as polymorphism; character-state optimization accelerated transformation (ACCTRAN). All characters were treated as unordered.

The branch and bound analysis produced 240 most parsimonious trees from which a strict (Fig. 11) and a 50% majority rule (Fig. 10) consensus tree was computed. These had the following characteristics in both analyses: tree length = 43; consistency index = 0.767; homoplasy index = 0.302; retention index = 0.839; rescaled consistency index = 0.644. Character state changes are included in Fig. 10.

If the search was made with multi-state taxa scored as uncertainty (to allow for the equivocal condition of the mitochondrial derivatives, character 9, in *Pogona*) an identical number of trees and topology were obtained but the tree was three steps shorter, with consistency index = 0.750; homoplasy index = 0.250; retention index = 0.839; rescaled consistency index = 0.629.

It must be stressed that the strict consensus tree (Fig. 11), agreed closely with the majority rule tree, differing only in placing *Cnemidophorus* on the same level as the remainder of its clade and *Lialis* on the same level as the *Carlia* and snake clades. These two polytomies correspond with the only percentages below 100% on the majority rule tree.

TABLE 2. — Comparative ultrastructure of spermatozoa of Squamata, *Sphenodon punctatus* and Chelonia. (Input data matrix).

Taxon	Characters 11111111 12345678901234567	Reference
Chelonia	00000000000000000	[26, 27]
Sphenodon punctatus	00000000000000000	[26, 27, 32]
Ctenotus robustus	10011001111101001	[37]
Chalcides ocellatus	1001?001131101001	[21]
Lacertidae	10011001501101001	[7, 11, 21]
Cnemidophorus sexlineatus	10012001111101001	[41, 42]
Tiliqua scincoides	10010001111101001	[35]
Carlia pectoralis	01112112201011101	[35]
Lampropholis delicata	01112112201011101	[37]
Heteronotia binoei	02010101401101001	[37]
Lygodactylus picturatus	02012101401101001	[21]
Lialis burtonis	0?112112301211011	[25, 37]
Pogona barbata	11012023111101001 3	[45]
Varanus gouldii	11012001121101001	[45]
Colubridae	00011212301101011	Serpentes:
Elapidae *(Oxyuranus)*	0001?212301101011	[3, 20, 21, 24, 33, 45, 46]
Boidae *(Aspidites)*	0001?212301101011	"
Iguanidae	00012002111001001 2 1	[22, 48]
Bradypodion karroicum	10012102201101001	This study

Discussion of the phylograms

The majority rule phylogram (Fig. 10) indicates those similarities of spermatozoal ultrastructure within the Squamata, and relative to *Sphenodon* and the Chelonia, which are synapomorphic for the respective nodes. These similarities, as in all spermiocladistic studies, must reflect similar fertilization biology but also phylogenetic, i.e. genetic, constraints. Spermatozoal morphology is here considered sufficiently conservative to reflect at least close phylogenetic relationship as it can be expected to be subject to stabilizing selection [22]. The evidence of many other studies (see, for instance, [26, 27, 31, 32] and references in [34]) shows that, although caution is required in their interpretation, such spermiocladistic phylograms hold significant phylogenetic information. Bearing in mind the necessity for caution and also the pressing need for inclusion of reliable data from a much larger sample of taxa, and confirmation of some data from the present sample, many of the following conclusions drawn from the phylogram can be regarded only as heuristic. Findings which both intuitively and by computation are unequivocal are stressed. Because of limitations of space, references to the literature will chiefly be confined to the excellent review of squamate classification by RIEPPEL [47].

Squamata. The Squamata, which are monophyletic in all most parsimonious trees in the analysis, are apomorphic relative to the Sphenodontida and Chelonia in the following features which are synapomorphies defining the Squamata s. strict. *Sphenodon* and the Chelonia are equally plesiomorphic and indistinguishable on the characters employed and therefore reference will chiefly be made to *Sphenodon*. It should be stressed, however, that *Sphenodon* is not here

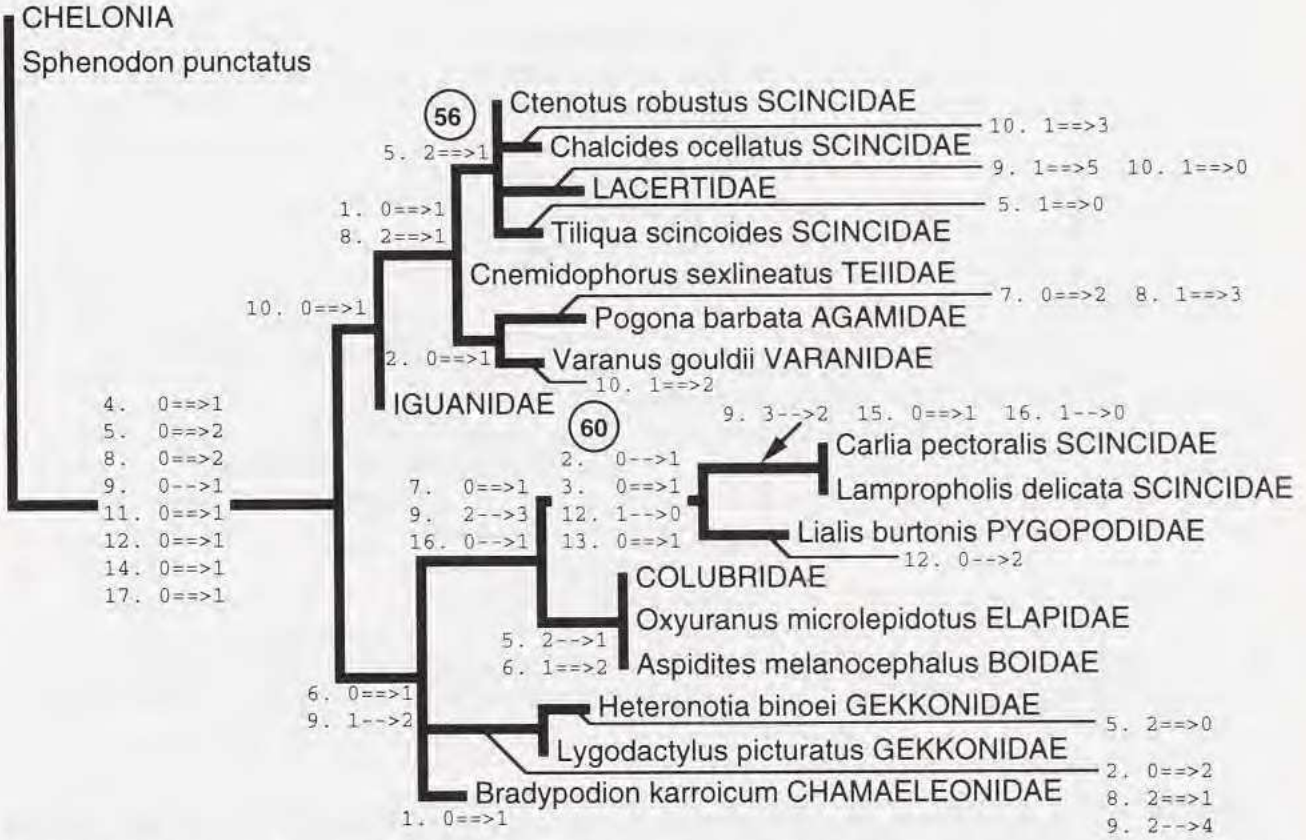


FIG. 10. — Branch and Bound 50 % majority rule consensus phylogram for 240 equally most parsimonious trees, for character states of chelonian, sphenodontid and squamate sperm ultrastructure listed in Tables 1 and 2. Settings as in text. Tree length = 43; consistency index = 0.767; homoplasy index = 0.302; retention index = 0.839; rescaled consistency index = 0.644. Numbers in circles are the percentage of trees supporting the node to which they attach. All other nodes were supported in 100 % of the trees.

regarded as the immediate sister-group of the Squamata *sensu strictu* but as a very basal amniote in a sphenodontidan lineage which possible predates emergence of the Crocodilia, Aves, and, it appears, the Mammalia [26, 32].

The squamate synapomorphies follow: 1. Possession of a single perforatorium in place of the two or three of Sphenodontida; 2. Loss of the endonuclear canal. The wholly epinuclear rather than partly endonuclear perforatorium is a notable difference of the Squamata from Chelonia, Sphenodontida, Crocodilia, non-passerine birds, and also basal lissamphibians (urodeles and *Ascaphus*). 3. Presence of the (well developed) epinuclear electron-lucent region. 4. Presence of sinuous mitochondria (possibly an artefactual parsimony resolution as a columnar form is intuitively preferred). 5. linear cristae, not the subspherical mitochondria, with concentric cristae of *Sphenodon*. 6. Dense bodies (mitochondrial transformations) intermitochondrial, in contrast with the intramitochondrial dense body in *Sphenodon*, and (equivocally) forming regular rings. Although the intermitochondrial position may well be basic to all squamates, one might query that the arrangement in regular rings is in their ground plan. 7. The fibrous sheath extends into the midpiece. This is a most significant and convincing synapomorphy and autapomorphy of the

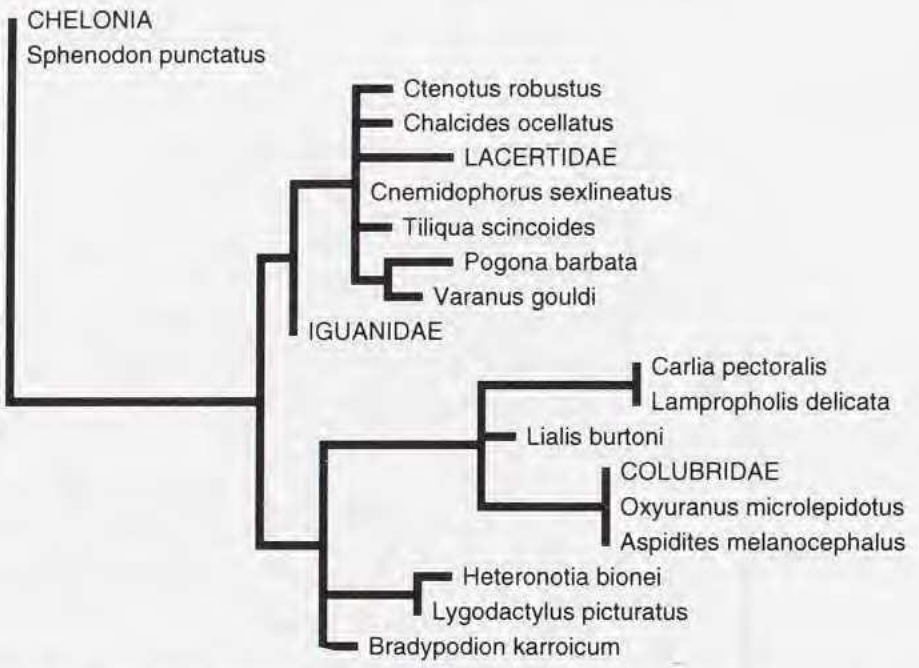


FIG. 11. — Branch and Bound 50 % strict consensus phylogram for 240 equally most parsimonious trees, for character states of chelonian, sphenodontid and squamate sperm ultrastructure listed in Tables 1 and 2. Settings, length and indices as in Fig. 10.

Squamata and is not seen in the Sphenodontida [26, 32]. 8. Development of rounded nuclear shoulders is basic to squamates. In addition, though not included in the parsimony analysis, squamates are diagnosed by the paracrystalline substructure of the subacrosomal cone.

These squamate spermatozoal autapomorphies constitute a striking endorsement of the "highly corroborated" monophyly of the Squamata for which evidence is reviewed by RIEPPEL [47].

'Sauria'. The Sauria is the third group of the Squamata, the other two being the Amphisbaenia and the Serpentes. Whereas the latter two groups appear to be monophyletic, RIEPPEL [47] stated that monophyly of the Sauria could not be established and that the name should be dropped or, in effect, broadened to equate with Squamata. If the evidence of the spermatozoal phylograms (Figs 10, 11) be accepted, the view that Sauria (in the strict sense of 'lizards' rather than squamates in general) are not monophyletic is emphatically endorsed as some saurians (invariably Eugongylus-group skinks and pygopodids, and, less closely, gekkonids and the chamaeleonid) appear more closely related to snakes than they are to other 'lizards'. Thus, Sauria is only monophyletic when it is, indeed, equated with Squamata and it includes lizards and snakes. The position of the amphisbaenians remains to be investigated when spermatozoal samples become available.

Scincomorpha. Monophyly of the Scincomorpha, though well supported by somatic morphology, has been considered to deserve further critical study [47]. The group is generally held to include the Xantusioidea, Lacertoidea (Lacertidae, Teiidae and Gymnophthalmidae) and the Scincoidea (Scincidae and Cordylidae). Spermatologically (Figs 10, 11) the Scincomorpha is diphyletic or possibly polyphyletic. This is due to the fact that the Scincoidea, represented in the parsimony analysis by the Scincidae only, are themselves at least diphyletic, notably because the Eugongylus-group skinks associate with the Serpentes, Pygopodidae, Chamaeleonidae and Gekkonidae

RIEPPEL (e.g. [47]) has repeatedly questioned the monophyly of the Scincidae and his doubts are underlined spermatologically. Thus the Eugongylus-group skinks (*Carlia* and *Lampropholis*) constitute the sister-group of the Pygopodidae; and the pygopodid-Eugongylus-clade is in turn the sister-group of the Serpentes in the majority rule tree (Fig. 10). However, the pygopodid forms a trichotomy with the Eugongylus-group and Serpentes clades in the strict consensus tree (Fig. 11).

The apparent synapomorphies joining the *Carlia* through Serpentes clades are weak. They are loss of the regular mitochondrial circlet, and, equivocally, development of linear dense bodies and of multilaminar membranes. Of these, the last two conditions are not seen in *Carlia* and *Lampropholis*. These two genera are linked to the pygopodid by four apomorphies. Two of these, the square-ended perforatorium and alteration of the nucleus to a stout form appear strong synapomorphies, while two are equivocal, acquisition of a knob-like basal plate (homoplasic with *Pogona* and *Varanus*) and development of sharp nuclear shoulders.

The severance of the Eugongylus-group from other skinks merits further investigation using non-spermatozoal characters. The spermatozoal synapomorphies defining the Eugongylus-group are striking. They are the characteristic development of scattered dense bodies, forming more than one layer around the axoneme; the fact that coarse fibres 3 and 8 are grossly enlarged anteriorly in addition to the usual enlargement in squamates; and, equivocally, reversion to no multilaminar cell membranes.

The position of the Lacertidae and Teiidae is poorly resolved (Fig. 10), and further data on these families are required. Linkage of the Lacertidae and Teiidae in the Lacertoidea [47] is not upheld. A teiid relationship to the Iguanidae (references in [47]) is also not supported. In the strict consensus tree (Fig. 11), lacertids, the teiid *Cnemidophorus*, and the skinks *Ctenotus*, *Chalcides*, and *Tiliqua* form an unresolved polytomy with the *Pogona-Varanus* clade. However, in the 50% majority rule tree (Fig. 10), *Cnemidophorus* is less close to the other scincomorphs than they are to each other. Intuitive considerations had placed teiids near sphenomorph (*Ctenotus*) and egernid (*Tiliqua*) skinks [37] but an especially close relationship is not upheld in this analysis.

Lacertid sperm have not been examined by the author but the composite data for the family, while placing them with the other, non-Eugongylus-scincomorphs, do not at present ally them more closely to any scincid or with the teiid. Their synapomorphies are arrangement of dense bodies into two groups and, of uncertain validity, reversal to a poorly developed epinuclear electron-lucent region. As recorded in the comparative section above, the arrangement of dense bodies in lacertids appears to be unusually diverse but is never strongly similar to the *Ctenotus* condition.

Iguania. Traditionally these include the Iguanidae, Anolidae, Tropiduridae, Agamidae and Chamaeleonidae. The Iguania does not emerge as a monophyletic group in the present analysis (Figs 10, 11). *Pogona* groups not with an iguanian but with *Varanus* in a clade which otherwise consists of non-Eugongylus scincomorphs while iguanids form the plesiomorph sister-group of this clade. The plesiomorphic status of the Iguanidae relative to agamids (references in RIEPPEL [47]) is endorsed but here in a paraphyletic relationship.

The chamaeleon Bradypodion is severed from the Iguanidae and is basal in the gekkonid-snake-pygopodid-Eugongylus-group clade in the majority rule (Fig. 10) and strict (Fig. 11) consensus trees in the present analysis. It may be noted, however, that in terms of patristic distance, the nearest taxon to the chamaeleon is, nevertheless, the Iguanidae. On intuitive consideration (see comparative account above) the sperm of the chamaeleon appear closely similar to that of the agamid in many respects but the sinuous tubular mitochondria are an important difference from agamids and, although a resemblance to iguanids, are also seen in the Carlia-pygopodid-snake clade. Much further investigation of spermatozoa, and comparison with morphological characters, is necessary if iguanian relationships are to be elucidated.

The knob-like basal plate appears to be the sole synapomorphy of *Varanus* and *Pogona* but is homoplasic with that in *Carlia* and *Lampropholis*.

Anguimorpha. Anguimorphs are conventionally divided into the Anguioidea and the Varanoidea [47]. Anguioidea have yet to be examined for sperm ultrastructure. In the comparative study above, the similarity of the *Varanus* sperm to that of *Pogona* has been described. From the comparative study, varanid sperm resemble those of agamids, sphenomorph and egernid skinks, and teiids in the depressed acrosome and intermitochondrial rings; they further resemble agamids and also (homoplasically) Eugongylus-group skinks in possessing a knob-like perforatorial base plate. Intuitively, as in the analysis (Fig. 10), the varanid is part of a sphenomorph-egernid-agamid assemblage. It is noteworthy that although a close relationships of varanids with scincids does not appear to have been previously suggested, brain data have been considered to indicate that the Teiidae are most closely related to the Varanidae [43].

A very close relationship between varanids and snakes which has formerly been postulated (see references in [45, 47]) is not supported by spermatozoal ultrastructure. *Varanus* sperm differ from those of snakes in the short midpiece, columnar mitochondria in a regular circlet, dense intermitochondrial bodies forming regular rings and absence of multilaminar membranes. It is expected that varanid relationships will come nearer to resolution when more than one species of each family is represented. A unique varanid apomorphy is replacement of the solid condition of the intermitochondrial rings, also seen in *Ctenotus*, *Cnemidophorus*, *Tiliqua*, *Pogona* and iguanids, with a granular condition.

Thus, the relationships of varanids remain enigmatic despite the parsimony analyses but a close relationship to snakes is not upheld.

Gekkota. Monophyly of the Gekkota, comprising the two extant families Gekkonidae and Pygopodidae has been considered well corroborated (references in RIEPPEL [47]). However, the majority rule consensus tree (Fig. 10) represents the Pygopodidae, exemplified by *Lialis burtonis*, as the sister-taxon of the Eugongylus-group skinks (for synapomorphies see Scincomorpha above). In the strict consensus tree (Fig. 11), *Lialis* forms a trichotomy with these skinks and the snakes. In the parsimony analysis (Figs 10, 11) the gekkonids form a monophyletic clade which, however, has an unresolved relationship with the chamaeleon, at the base of the snake+pygopodid+Eugongylus-group clade. The only unequivocal synapomorphy for the composite clade, is development of a moderately long midpiece which becomes very long in snakes. No unique synapomorphies are apparent between the sperm of gekkonids and pygopods which would support the special relationship between these two families suggested by KLUGE [38, 39, 40] who from a cogent cladistic study of general morphology has concluded [39] that the Pygopodidae should be placed within the Gekkonidae. This departure from somatic morphological evidence requires further investigation from sperm of larger numbers of taxa.

A relatively primitive position of the Gekkonidae in the 'Sauria' proposed by some workers [47], as opposed to the Pygopodidae, is supported by both consensus phylograms. Gekkos have somewhat generalized scincid sperm but a stellate arrangement of dense bodies in the midpiece distinguishes at least some of them. *Heteronotia* and *Lygodactylus* are sister-taxa in 100% of the trees. Computed synapomorphies of gekkonids (*Heteronotia* and *Lygodactylus*) are the stopper-like perforatorial base, the columnar condition of the mitochondria, supposedly derived secondarily from a sinuous condition; and, ambiguously, the stellate mitochondrial derivatives which compute as arising from a scattered condition such as is seen in Eugongylus-group skinks.

Serpentes. The origin of snakes, on the grounds of somatic morphology, continues to be an enigma [47]. However, the spermiocladistic analysis indicates origin of snakes from an ancestor, which must have been skink-like, shared with Eugongylus-group skinks (*Carlia* and *Lampropholis*) and with pygopodids. The pygopodid has a sister-group relationship with the Eugongylus-group skinks in the majority rule tree (Fig. 10) but forms an unresolved trichotomy with these and snakes in the strict consensus tree (Fig. 11). At present this origin of snakes must remain merely an heuristic finding but spermatozoa do provide a serpent apomorphy (see also [33, 45]) shared only with the pygopodid, presence of multilaminar sperm cell-membranes. These

membranes appear much better developed in snake sperm which differ further, and uniquely, in the immense elongation of the midpiece as a mitochondrial sheath around most of the length of the axoneme. That the multilaminar membranes have been lost in Eugongylus-group skinks, as computed, may be questioned. If not, presence would be a snake-pygopod synapomorphy. Further snake apomorphies appear to be reduction of the epinuclear electron-lucent region and loss of a perforatorial base plate. Snake sperm also have a greater development of extracellular tubules (not coded) than is known in any other squamate.

With regard to groups not included in the analyses, tropidurid sperm are insufficiently known for determination of phylogenetic affinities but the three gyres of mitochondria are also a lacertid characteristic; supposed failure of the fibrous sheath to penetrate the midpiece (the squamate autapomorphy) requires confirmation.

Investigations of sperm of additional taxa are required if suggested relationships throughout the Squamata are to be adequately tested.

ACKNOWLEDGEMENTS

I am grateful to Mrs Lina DADDOW, David SCHELTINGA, Chris TUDGE, and Simon OLIVER for excellent assistance and to Chris TUDGE and Tom GORRINGE for printing the micrographs. Professor Alan HODGSON and Dr. Rick BERNARD kindly supplied a resin block of *Bradypodion* sperm. This work was partly supported by an Australian Research Council grant.

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